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PRODUCTION, CHARACTERIZATION AND USE OF MONOCLONAL ANTIBODIES TO PROTEODERMATAN SULFATE

by

GORDON ANTHONY PRINGLE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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IN

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EDMONTON, ALBERTA

FALL, 1985

ERRATA

1. Page 72. Insert at bottom:

..."proteoglycan monomer was blotted that it prevented nonspecific staining at that position and was visible as a white, unstained image. All of the antibodies were inhibited to some extent by bovine nasal cartilage A1D1 and its (ABC)core preparation (Fig. 14). However, immunoblots....."

2. Page 77. Delete from:

..."immunoblots (Plate 21). However, all 4 antibodies ...." to end of page.

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DEDICATION

I dedicate this thesis to my most patient and caring friend, my wife Jeanine and to our lovely daughter Caitlin.

ABSTRACT

Proteodermatan sulfate, a small proteoglycan containing L-iduronic acid-rich dermatan sulfate side chain(s), is synthesized by fibroblasts and has been extracted from dense connective tissues rich in type I collagen. Based on a number of studies it has been proposed that this proteoglycan is closely associated with type I collagen and functions to regulate collagen fibrillogenesis and fibril growth, collagen turnover and calcification.

To study the molecular structure and function of bovine skin proteodermatan sulfate, on a determinant by determinant basis, several monoclonal antibodies to this molecule have been produced and characterized. Based on the results of a preliminary immunogenetic analysis of 4 inbred mouse strains, SJL/J (H-2s) mice were immunized for the fusions. Ten hybrid cell lines were produced and four of these were subsequently recloned to monoclonal status. Ascites and supernatant fluids were obtained from each of the clones and the antibodies present in each were investigated further.

Employing an ELISA inhibition assay, none showed any detectable affinity for bovine collagen types I-IV, bovine fibronectin, elastin, chondroitin or dermatan sulfate, nor were they inhibited by the link proteins or the proteoglycan monomer from bovine nasal cartilage or the large chondroitin sulfate-containing proteoglycan from dental pulp. Each monoclonal antibody recognized the protein core of proteodermatan sulfate after treatment with anhydrous HF and none was significantly inhibited by proteolytically degraded proteodermatan sulfate suggesting that their epitopes have a protein component. The results of competitive binding ELISA assays, immunoblots of the CNBr cleavage products of proteodermatan sulfate and ELISA inhibition assays with the chondroitinase ABC-derived protein core of the proteoglycan indicated that the 4 antibodies recognize at least 3 distinct epitopes on this molecule. Antibodies 1XA and 6D6 may recognize a single epitope or 2 adjacent epitopes within a large CNBr peptide from near the C-terminal of the protein core. The epitope of 7B1 is species specific and resides near the site of attachment of the single dermatan sulfate side chain at residue 4, probably between residues 9 and 20. Recognition by all 4 monoclonal antibodies of various fragments of the protein core of

proteodermatan sulfate on immunoblots suggests that the epitope on each is sequential, rather than conformational, in nature.

Employing immunoblotting methods, the monoclonal antibodies have revealed that the protein core of proteodermatan sulfate may possess a variable number of N-linked oligosaccharides that is species and tissue dependent. The antibodies also revealed a structural similarity between proteodermatan sulfates from various tissues and that differences in their size were largely due to differences in their content of glycosaminoglycan. Recognition by several of the monoclonal antibodies of a small proteoglycan in culture fluid of monkey arterial smooth muscle cells suggests that the same protein core may possess an L-iduronic acid-rich or an L-iduronic acid-poor dermatan sulfate side chain.

Light microscopic immunohistochemical methods located proteodermatan sulfate in the connective tissue of dermis and several other tissues and also indicated that the position of the antigen in the dermis changes during skin development. Immunoelectron microscopic studies of bovine tail tendon have indicated that the protein core of proteodermatan sulfate is associated with type I collagen fibrils in the vicinity of the *d* and *e* bands, possibly between them, suggesting that the protein core possesses a specific collagen fibril binding site. The functional significance and implications of this result, as it relates to the function and organization of collagen fibrils, are discussed.

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LIST OF ABBREVIATIONS

Abbreviations used in this manuscript are:

Mr:	relative molecular mass
CS:	chondroitin sulfate
GAG:	glycosaminoglycan
PDS:	proteodermatan sulfate containing L-iduronic acid-rich dermatan sulfate side chains.
(ABC)core:	the protein core of a proteoglycan that results following digestion with chondroitinase ABC
HABR:	hyaluronic acid binding region
MAbs:	monoclonal antibodies
GdnHCl:	guanidine hydrochloride
CNBr:	cyanogen bromide
CPC:	cetylpyridinium chloride
PMSF:	phenylmethanesulfonyl fluoride
NEM:	N-ethylmaleimide
EDTA:	ethylenediamine tetraacetate
SDS:	sodium dodecyl sulfate
H-2:	the major histocompatibility gene complex of the mouse
ELISA:	enzyme linked immunosorbent assay
PG1:	the uronic acid-containing fraction that elutes near V_o on Sepharose CL-4B columns
PG2:	the uronic acid-containing fraction that elutes as peak 3 on Sepharose CL-4B columns

A1D1: the high buoyant density fraction of a cartilage extract purified by CsCl density gradient centrifugation under associative and dissociative conditions.

A1D4: the low buoyant density fraction of a cartilage extract purified as described for A1D1

LH: lactalbumin hydrolysate

FCS: fetal calf serum

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

PBS/T: phosphate buffered saline containing Tween 20

TBS: Tris buffered saline

PEG: polyethylene glycol

NGS: normal goat serum

NMS: normal mouse serum

(BES)core: the protein core of a proteoglycan that results following a β-elimination, sulfite addition reaction.

(HF)core: the protein core of a proteoglycan that results following treatment with anhydrous HF.

PAS: periodic acid-Schiff stain

S.D.: standard deviation

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1. INTRODUCTION

1.1 The Form and Function of Connective Tissue Matrices

Based on shared morphological and functional characteristics, all tissues of the vertebrate body may be classified as belonging to one of four primary types; connective tissue, epithelium, muscle and nerve (Leeson and Leeson, 1976). Connective tissues are characterized by an extensive extracellular matrix that consists largely of fibers and an amorphous ground substance, the nature and proportion of each varying from one type of connective tissue to the next. Connective tissue matrices are present in the adult in many architectural forms, dictated largely by the phenotype of their respective biosynthetic cells (see Table 1) and although their basic structural elements dictate that they all function primarily as scaffolds of varying consistency that physically support and bind together the more organized tissues of the body, many other important functions have also been attributed to them (Le Gros Clark, 1971). To understand how these different connective tissue matrices function physiologically and malfunction pathologically, it is necessary to gain a more complete understanding of their macromolecular components and how they interact with one another.

1.2 The Fibrous Components of Connective Tissue Matrices

The fibrous elements in connective tissue are those in which the constituent macromolecules do not function individually, but as highly ordered polymers. Their aggregation into larger structures appears to be an inherent tendency, based on their molecular structure, and therefore must of necessity be a process under strict biological control. The two most important fibrous components of connective tissue are collagen and elastin.

1.2.1 Elastin

Elastin is present in many connective tissues. Insoluble fibrous elastin is composed of elastin monomers (Mr = 68,000) that are rich in hydrophobic residues, especially Gly, Ala, Val

TABLE 1
CONNECTIVE TISSUES OF THE BODY

Class	Type	Subtype	Biosynthetic Cell	Example
General	Loose	Mesenchyme	Fibroblast	Embryo
		Mucoid	Fibroblast	Umbilical cord
		Areolar	Fibroblast	Areolar tissue
		Adipose	Fibroblast	Subcutaneous tissue
		Reticular	Fibroblast	Bone marrow
	Dense	Irregular	Fibroblast	Dermis
		Regular	Fibroblast	Tendon, cornea
Special	Cartilage	Hyaline	Chondroblast	Nasal septum
	Bone		Osteoblast	Alveolar bone
	Dentin		Odontoblast	Tooth
	Cementum		Cementoblast	Tooth

and Pro. These amino acids occur in the molecule in a highly organized manner generating three structurally distinct domains consisting of repeating (Val-Pro-Gly-Val-Gly) pentamers and (Ala-Pro-Gly-Val-Gly-Val) hexamers, and an alanine-rich α helix. Each pentamer and hexamer are capable of forming a β turn and the contiguous pentamers and hexamers in their respective domains form structurally distinct β spirals (Urry et al., 1974; Urry and Ohnishi, 1974) The hexameric β spiral is short and rigid and interacts with the same spiral on adjacent tropoelastin molecules to align them to allow formation of bi- and tetra-functional intermolecular crosslinks derived from modified lysine residues (Thomas et al., 1963; Franzblau et al., 1965). The longer, terminally situated pentameric β spirals of 3 adjacent molecules entwine about each other to form an open, water filled helix, that is proposed to be the primary structural feature responsible for the entropic elastic qualities of fibrous elastin (Urry, 1983).

1.2.2 Collagen

Collagen, in its many forms, is by far the most abundant fibrous connective tissue protein in the vertebrate body. Collagen monomers consist of 3 polypeptide chains (i.e., α chains) that in turn consist largely of the repeating amino acid triplet (Gly-X-Y) which permit the 3 α chains to coil tightly about one another into a distinctive triple helix (Ramachandran and Kartha, 1955). Unlike the open, spring-like spirals of elastin, collagen monomers are typically long and rod-like and, when polymerized into fibrils stabilized by covalent intermolecular crosslinks, endow tissues with increased tensile strength. Five distinct types of collagen (I-V) have been isolated and well characterized, although many other collagen-like molecules are currently under study (Table 2).

1.2.2.1 Biosynthesis of Type I Collagen

Most of what is known of the biochemistry of collagen has been obtained from studies of type I collagen and it serves as the standard for comparison. This type is present in many tissues and organs of the body, but is most prominent in the very fibrous connective tissue matrices of bone, cementum, dentin, adult dermis, tendon, sclera and the capsules of organs. Its biosynthesis is complex and is initiated by transcription of the large (38 kb) proα1(I) and proα2(I) genes (Wozney et al., 1981; De Crombrugghe et al., 1982) each containing many exons of 54 bp (or multiples of 9 bp) separated by introns of varying length. Translation of the 5 kb mRNAs on membrane-bound polysomes of the rough endoplasmic reticulum results in proα1(I) and proα2(I) polypeptide chains having 3 structural domains: a central "collagenous" domain consisting of 338 triplets of (Gly-X-Y) flanked at both ends by large, mostly globular N- and C-terminal propeptides. Susceptible proline and lysine residues are hydroxylated co-translationally by specific enzymes and several of the newly formed hydroxylysines are in turn enzymatically glycosylated with glucosyl-galactosyl disaccharides. The juxtaposition and alignment of the appropriate proα chains (two proα1 and one proα2), thought to be controlled by the association and disulfide cross-linking of their respective C-terminal propeptides (Rosenbloom et al., 1976), permits

TABLE 2
STRUCTURE AND DISTRIBUTION OF COLLAGENS[1]

TYPE	CHAINS	MOLECULAR SPECIES	DISTRIBUTION	REFERENCE
I	α1(I), α2(I)	α1(I),α2(I)	Virtually ubiquitous	Gay and Miller, 1983
II	α1(II)	α1(II),	Hyaline cartilage	Miller & Matukas, 1969
III	α1(III)	α1(III),	Distensible tissues	Miller et al., 1971
IV	α1(IV), α2(IV)	Unknown	Basement membranes	Timpl & Martin, 1982
V	α1(V), α2(V), α3(V)	α1(V),α2(V)	Pericellular locales	Burgeson et al., 1976
"I Trimer"	Embryonic α1	α1("E"),	Embryonic tissue	Shupp-Byrne et al., 1982
"Type VI"	40A, 40B,, 40B,	Unknown	Blood vessel walls	Furuto and Miller, 1980
"Type M"	33K	33K,?	Hyaline cartilage	Shimokomaki et.al., 1980
"Type CF1"	25K	25K,?	Hyaline cartilage	Shimokomaki et al., 1981
"Type CF2"	12K	12K,?	Hyaline cartilage	Shimokomaki et al., 1981
EC	EC-1	EC,?	Cultured endothelial cells	Sage et al., 1980
Random coil	RC	Unknown	Cultured endodermal cells	Engvall et al., 1982
Unknown	1α, 2α, 3α	Unknown	Hyaline cartilage	Burgeson et al., 1982
Unknown	59K	Unknown	Cultured chondrocytes	Gibson et al., 1982

[1]Adapted from Gay, S. and Miller, E.J. (1983) Ultrastructural Pathology, 4: 365-377.

their collagenous domains to spontaneously coil about one another to form the triple helical coiled coil. The hydroxyproline residues, present at the Y position in about 10% of the (Gly-X-Y) triplets, help to stabilize the nascent helix by hydrogen bonding with water molecules to form intramolecular water bridges (Ramachandran et al., 1973). The newly formed procollagen molecules are subsequently carried to the Golgi complex (Nist et al., 1975) where they are ultimately packaged in secretory granules for transport to the cell surface (Weinstock and Leblond, 1974; Cho and Garant, 1981). As visualized by electron microscopy, the procollagen molecules in the granules are arranged more or less side by side in aggregates (Bruns et al., 1979; Trelstad and Hayashi, 1979; Cho and Garant, 1981).

1.2.2.2 Fibrillogenesis of Type I Collagen

The manner in which collagen molecules are believed to polymerize to form fibrils has been derived from numerous *in vitro* experiments. Turbidimetric studies of the kinetics of fibril formation using purified soluble collagen molecules have indicated that an initial lag phase occurs during which collagen monomers aggregate to form long, thin filaments (Gelman and Piez, 1980; Silver and Trelstad, 1979). It has been proposed that a critical early step in the process is the formation of dimers and trimers aligned in a 4D stagger (Silver et al., 1979; Fig. 1). Further growth may follow by the stepwise D periodic aggregation of larger subassemblies (Silver, 1981; Silver, 1982; Trelstad, 1982). Stabilization of these subassemblies appears to rely heavily upon the N- and C-terminal, non-helical telopeptides (Comper and Veis, 1977; Helseth and Veis, 1981; Silver, 1982), which ultimately participate in the formation of lysine-derived intermolecular crosslinks stabilizing the 4D stagger within the fibril (Scott, 1980).

The extent to which the stepwise model of fibrillogenesis accurately reflects the events *in vivo* remains to be determined. It has been suggested that the intracellular aggregates of procollagen may be D periodic (Birk and Trelstad, 1984) and may therefore be in some intermediate form of aggregation consistent with that proposed by the model described above. Once released at the cell surface, probably in a deep recess near the site of

Fig. 1. Collagen fibril formation, based on the model proposed by Trelstad et al. (1982). Individual collagen molecules participate in the formation of dimers and trimers which aggregate to form small subassemblies. The growth phase of fibrillogenesis *in vitro* occurs as these structures aggregate to form larger subassemblies. The fibril is formed by this stepwise aggregation to ever larger structures.

Fig. 2. Diagrammatic representation of the 2-dimensional molecular arrangement of tropocollagen molecules according to Petruska and Hodge (1964). The molecules are aligned parallel to one another in a fibril, but staggered laterally with respect to one another by 67 nm, or 1 D period. Each molecule is 4.4 D in length; there being a 0.6 D gap between the N and C terminals of consecutive molecules. Laterally, each D period consists of all 5 regions of a collagen molecule; every 5th containing the gap region.

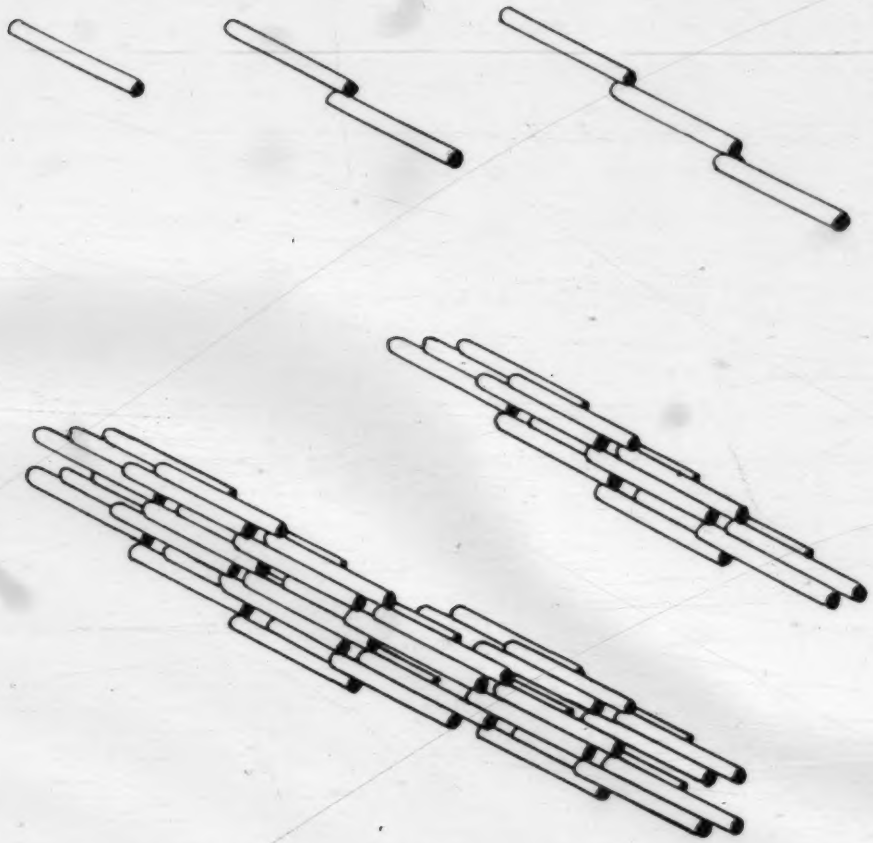

Figure 1.

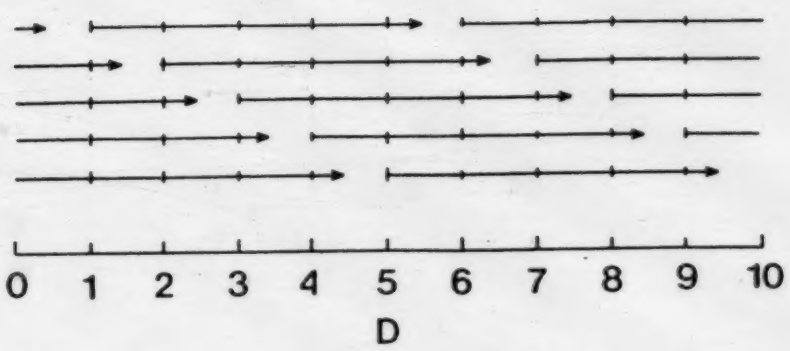

Figure 2.

extracellular fibril formation (Trelstad and Hayashi, 1979; Birk and Trelstad, 1984), the procollagen molecules are eventually processed by specific N and C propeptide proteases (Leung et al., 1979) and can then proceed to form dimers and trimers. If they already exist in this orientation as a result of intracellular aggregation, they can presumably aggregate further. Recently however, it has become apparent that the order and the timing with which the propeptides are excised may play a crucial role in fibrillogenesis and fibril morphology. Kinetic studies of fibrillogenesis *in vitro*, in conjunction with electron microscopy, have revealed that pN collagen molecules (collagen molecules with only the N-terminal propeptide) form thin fibrils, slowing lateral aggregation of molecules (Miyahara et al., 1983) while pC collagen molecules (collagen molecules with only the C-terminal propeptide) promote lateral aggregation and give rise to thick fibrils (Miyahara et al., 1984). In keeping with this, the occurrence of pN collagen in type I collagen fibrils *in vivo* seems to be restricted to those which have been recently synthesized and are relatively thin; 10-35 nm in diameter as seen in embryonic tissue (Fleischmajer et al., 1983). Larger diameter fibrils in more mature tissue did not have the N-terminal propeptide associated with them and the possibility that further increases in fibril diameter occur as a result of fibril fusion has been suggested (Scott et al., 1981; Fleischmajer et al., 1983). The presence of N-terminal propeptides on the surface of growing fibrils *in vivo* may play a role not only in controlling fibril diameter (Hulmes, 1983) but fibril production as well by their ability, once excised, to inhibit the translation of proα(I) mRNA (Hörlein et al., 1981). Other intermediates (i.e., pC collagen) have been detected in cultured bovine smooth muscle cells (Gersternfeld et al., 1984) and chick cranial bones (Davidson et al., 1977), suggesting the possibility that cells can regulate the processing pathway according to the needs of the matrix.

In two dimensions within a fibril, the collagen molecules are believed to be related to one another by a 1D stagger (Fig. 2). As each monomer is 4.4D in length, a gap is present between the N and C terminals of axially related monomers. The three dimensional

organization of collagen monomers in a fibril remains unknown but X-ray diffraction data (Hulmes and Miller, 1979; Piez and Trus, 1981) and electron microscopic findings (Hulmes et al., 1981) indicate that the monomers are packed within fibrils in a quasi-hexagonal array.

1.2.2.3 The Other Collagens

The biosynthesis of the other major collagens is thought to proceed in the same general manner as type I. Differences occur in the extent to which their proline and lysine residues are hydroxylated and in their content of glucosyl-galactosyl disaccharides (see Table 2, Nimni, 1983).

Type III collagen molecules are present in many of the same tissues as type I collagen, at times crosslinked to one another (Henkel et al., 1981). Type III collagen fibrils are usually fine in diameter. This may be due to the retention of at least part of its N-terminal propeptide (Nowack et al., 1976; Fessler et al., 1981).

Type II collagen molecules contain more of the bulky, hydrophilic glucosyl-galactosyl disaccharides and form more hydrated fibrils (Grynpas et al., 1980). Not coincidentally, type II is present in hydrated, gel-like matrices: hyaline cartilage, nucleus pulposus and the vitreous of the eye. In contrast to the fine fibrils observed *in vivo*, type II collagen fibrils formed *in vitro* are much larger and while some are D-periodic structures, others are tactoids or structureless filaments (Lee and Piez, 1983) suggesting that type II collagen fibril formation *in vivo* may be influenced by other components of the extracellular matrix (Lee and Piez, 1983; Parry et al., 1981; Hjelle and Gibson, 1979).

Type V collagen is found in a variety of tissues in close association with cell surfaces (Bailey et al., 1979; Gay et al., 1981a; Gay et al., 1981b), and it has been proposed that type V collagen is a component of the exocytoskeleton (Gay et al., 1981a). Isolated from lathyritic chick bone (Broek et al., 1985), type V collagen possesses a large globular domain at one of the triple helix and is capable of forming small-diameter fibrous structures.

Type IV collagen contains many disaccharide side chains and retains both its N- and C-terminal propeptides and does not function in the form of fibrils. Instead, the N propeptide self-associates with the respective propeptides of 3 other molecules and forms a disulfide bonded tetramer (Duncan et al., 1983). The C propeptide of the molecule participates with those of 3 other molecules also forming a tetramer. In this fashion type IV collagen molecules form an open lattice, an ideal supporting framework for porous basement membranes (Timpl et al., 1981).

1.3 The Non-fibrous Components of the Extracellular Matrix

Although collagen fibrils are probably formed and metabolically turned over within a controlled milieu adjacent to the cell surface (Trelstad and Hayashi, 1979) and much of fibrillogenesis may depend on the presence or absence of the propeptides, the process of collagen assembly may nonetheless be influenced by other components of connective tissue synthesized simultaneously by the cell or derived from the interstitial fluid. Certainly, once formed, the collagen fibrils become integrated into the extracellular matrix by closely associating with a number of non-fibrous components such as glycoproteins, hyaluronic acid and proteoglycans.

1.3.1 Connective tissue Glycoproteins

Glycoproteins are, by definition, proteins with a variable number of oligosaccharide side chains. The oligosaccharides may be O-linked through N-acetylgalactosamine to serine or threonine residues or N-linked through N-acetylglucosamine to the side chain amide nitrogen of asparagine residues. The N-linked oligosaccharides are synthesized in the form of a dolichol-phosphate linked precursor (Li et al., 1978) and subsequently transferred to a susceptible Asn residue (in the sequence Asn-X-Ser/Thr) of the protein in the rough endoplasmic endothelium. The N-linked oligosaccharides are further modified in the smooth endoplasmic reticulum and Golgi complex. The O-linked oligosaccharides are added directly to the protein in the Golgi complex. These oligosaccharides are attached to exposed surfaces of the

protein core and function to increase its hydration and solubility and/or render that region of the protein core less susceptible to proteolysis. They may also serve as sites of interaction with other molecules. The major glycoproteins found in connective tissues may be classified on the basis of function, but all appear to have one or more binding sites that specifically recognize other connective tissue macromolecules.

1.3.1.1 The Adhesive Glycoproteins

The so-called "adhesive" glycoproteins facilitate the attachment of cells to an adjacent collagen substratum. The most thoroughly studied of these is fibronectin (Ruoslahti et al., 1982) Located at the cell surface as a disulfide bonded multimer (McKeown-Longo and Mosher, 1984), multifunctional fibronectin molecules serve as a molecular bridge between the cell surface glycocalyx of fibroblasts and types I and III collagen fibrils. Vitronectin, an adhesive glycoprotein consisting of 65,000 and 75,000 dalton polypeptide chains has recently been isolated from human plasma and like fibronectin possesses a heparin binding site and promotes adhesion and spreading of fibroblasts (Suzuki et al., 1984). Chondronectin attaches chondrocytes to native type II collagen fibrils in hyaline cartilage (Hewitt et al., 1982) and laminin attaches epithelial cells to the type IV collagen meshwork in basement membranes (Terranova et al., 1980). Entactin, a sulfated glycoprotein in basement membranes assists laminin in epithelial cell adhesion (Martinez-Hernandez and Chung, 1984).

1.3.1.2 Structural Glycoproteins

Other glycoproteins function to bind connective tissue macromolecules together into larger structures or aggregates. The most thoroughly characterized of these are the link proteins. First discovered in hyaline cartilage extracts (Hascall and Sajdera, 1969), these globular molecules (Baker and Caterson, 1979; Lé Glédic et al., 1983; Poole et al., 1984) function to stabilize the interaction between the large cartilage proteoglycan monomer and hyaluronic acid (Caterson and Baker, 1979; Kimata et al., 1982a) although they may also

attach the fibrillar interstitial collagens to large proteoglycans, hyaluronic acid or their aggregates (Chandresekhar et al., 1983; Chandresekhar et al., 1984). A number of other glycoproteins have also been isolated with cartilage proteoglycan aggregates (Kleine and Singh, 1982) although their function remains obscure. Other "acidic structural glycoproteins" have been isolated from aorta (Moczar et al., 1967), heart valve (Goldstein et al., 1967), skin and joints (Timpl et al., 1968; Timpl et al., 1969) but remain poorly defined. It has been proposed that these molecules serve to bind collagen fibrils into fiber bundles. Osteonectin (Termine et al., 1981) binds hydroxyapatite to collagen fibrils.

1.3.2 Hyaluronic Acid

A significant proportion of the amorphous, interfibrillar ground substance in virtually all of the connective tissue matrices is hyaluronic acid. It is a high molecular weight (160,000 to 1,300,000 daltons in rabbit skin; Shimada and Matsumura, 1977) (500,000 daltons in bovine nasal cartilage; Heinegård et al., 1978) non-sulfated, acidic glycosaminoglycan consisting exclusively of the repeating disaccharide, D-glucuronic acid--N-acetylglucosamine (Fig. 3). It lacks covalently bound protein (Sugahara et al., 1979) and is synthesized by a hyaluronate synthetase system thought to be located in the external surface of the plasma membrane of cells (Philipson and Schwartz, 1984; Rodén et al., 1985). By virtue of its size and negative charge, hyaluronic acid is believed to form an entangled network possessing an extremely large hydrodynamic volume (Ogston and Stanier, 1953) enabling it to behave as a viscoelastic gel (Gibbs et al., 1968). The properties of the gel (i.e., the ground substance) change according to the concentration of hyaluronic acid and the presence of collagen (Fessler, 1960) and large proteoglycans (Disalvo and Schubert, 1966). Interactions between these components may be mediated indirectly by the link proteins discussed above or directly by weak, noncovalent forces (Mathews, 1965). In addition to its role in the ground substance hyaluronic acid also appears to play a key role in the detachment and mobility of cells on collagen substrates (Lark and Culp, 1982; Sommarin and Heinegård, 1983).

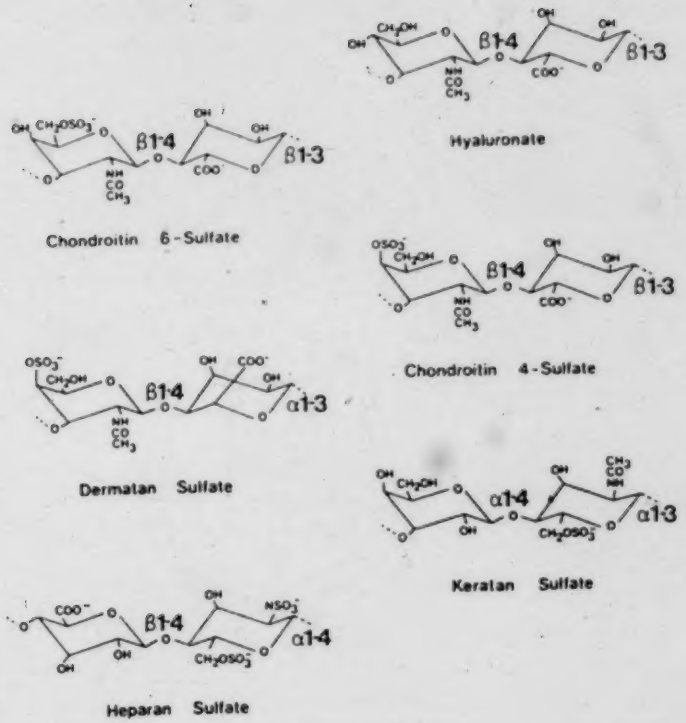

Fig. 3. Major disaccharides present in each type of glycosaminoglycan. Hyaluronic acid consists of repeating (N-acetylglucosamine-D-glucuronic acid) disaccharides. The chondroitin sulfates (CS) consist of repeating (N-acetylgalactosamine-D-glucuronic acid) disaccharides and vary in the position in which they are substituted with sulfate. Chondroitin sulfate side chains usually consist of mixtures of C4S and C6S. Dermatan sulfate disaccharides are biosynthetically derived from the chondroitin sulfate disaccharides (usually C4S) by epimerization of D-glucuronic acid residues to L-iduronic acid. As epimerization occurs at the polymer level and is not complete, dermatan sulfate side chains retain a proportion of chondroitin sulfate-like, D-glucuronic acid-containing disaccharides. Dermatan sulfate side chains are therefore described as L-iduronic acid-rich or L-iduronic acid-poor. The C-2 position of L-iduronic acid residues may also be sulfated. Heparan sulfate side chains are similar to those of dermatan sulfate in that they also contain a proportion of L-iduronic acid residues, derived from a similar epimerization reaction. A high rate of epimerization is associated with increased sulfation and the formation of heparin.

1.3.3 Proteoglycans

Proteoglycans are structurally complex macromolecules located on the surface of collagen fibrils, in the interfibrillar spaces with hyaluronic acid forming the ground substance and pericellularly as part of the glycocalyx. They resemble glycoproteins in that they consist of a core glycoprotein with a variable number of N- and O-linked oligosaccharides (Nilsson et al., 1982; Thonar et al., 1983; Shinkai et al., 1983), but differ in that they also possess a variable number of high molecular weight polyanionic glycosaminoglycan side chains which, like hyaluronic acid, consist of repeating disaccharide units (see Fig. 3). These carbohydrate side chains differ primarily in the constituents of their disaccharide units, the glycosidic linkages within and between disaccharide units and the number and position of their charged groups. Unlike hyaluronic acid, these glycosaminoglycans are not structurally homogeneous. Each disaccharide unit may contain 0, 1 or 2 sulfate groups. The chondroitin sulfate glycosaminoglycans often contain both chondroitin 4- and 6-sulfate disaccharides and both heparan sulfate and dermatan sulfate glycosaminoglycans are copolymers consisting of disaccharides containing either D-glucuronic acid or L-iduronic acid, the latter derived by epimerization of D-glucuronic acid residues by a specific C-5 epimerase (in heparan sulfate, Lindahl and Bäckström, 1972; in dermatan sulfate, Malmström et al., 1975; Malmström, 1984) Like the oligosaccharides, these large glycosaminoglycan side chains undoubtedly serve to increase the solubility of the proteoglycan and, depending upon their size and number, greatly increase its hydrodynamic size as well. However, the existence of different types of glycosaminoglycans and their structural microheterogeneity strongly suggest that they possess different conformations and are capable of unique interactions with other extracellular components (see below).

1.3.3.1 The Cartilage-Specific Proteoglycan Monomer

The most thoroughly studied proteoglycan is the large (1,200,000-2,500,000 daltons; Shogren et al., 1983) "monomer" present in hyaline cartilages (Sajdera and Hascall, 1969), schematically illustrated in Fig. 4. This proteoglycan is synthesized by

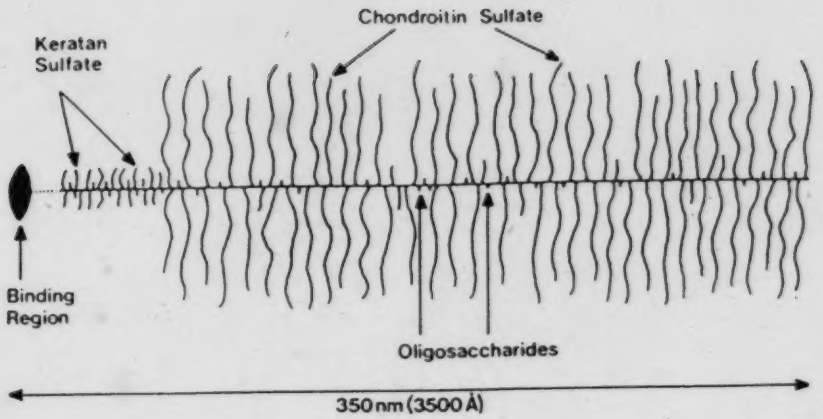

Fig. 4. Diagrammatic representation of the structure of the large proteoglycan "monomer" present in cartilaginous tissues. The "binding region" refers to the globular, non-glycosaminoglycan-bearing region of the molecule that possesses binding sites for hyaluronic acid and the link proteins. Adapted from Perkins et al. (1981).

chondroblasts and chondrocytes (Goetinck et al., 1974; Okayama et al., 1976) and is unique in that it contains two distinct types of glycosaminoglycan side chains. The protein moiety of the monomer from bovine nasal cartilage (M.W.=210,000 daltons; Olson, 1982) is divided into three regions that differ in structure and function: a) a chondroitin sulfate-rich region bearing 80-100 chondroitin sulfate chains that vary in molecular weight (Mr=15,000-20,000) and are attached to the protein core via a short tetrasaccharide (D-glucuronosyl-galactosyl-galactosyl-xylosyl) O-glycosidically linked to serine residues (Lindahl and Rodén, 1966); b) a shorter keratan sulfate-rich region of extended conformation bearing smaller keratan sulfate chains O-glycosidically linked to serine or threonine residues via N-acetylgalactosamine; and c) a globular, (Mr=60,000) non-glycosaminoglycan-bearing region that has a binding site specific for a decasaccharide

unit of hyaluronic acid (Christner et al., 1979). The large number of glycosaminoglycan side chains attached to this proteoglycan give it a large hydrodynamic volume and *in vivo*, many of these proteoglycan monomers are bound (and stabilized by link proteins) to a single hyaluronic acid molecule forming huge hydrated and entangled aggregates of very high molecular weight (Hardingham and Muir, 1972; Shogren et al., 1983). Together with the network of type II collagen fibrils, these aggregates endow cartilage, particularly articular cartilage, with a high degree of viscoelasticity and an ability to withstand deformation and recover its shape after pressure has been applied (Kempson et al., 1970).

1.3.3.2 Other Proteoglycans

Other large proteoglycans capable of aggregating with hyaluronic acid have also been isolated from aorta (Salisbury and Wagner, 1981; Wagner et al., 1983) and culture medium of arterial smooth muscle cells (Wight and Hascall, 1983; Chang et al., 1983). The very large chondroitin sulfate proteoglycan synthesized by embryonic chick skeletal muscle (Carrino and Caplan, 1982; Carrino and Caplan, 1984) may also aggregate with hyaluronic acid. These proteoglycans are as large or larger than the cartilage-specific monomer, but do not contain keratan sulfate and have fewer, but longer chondroitin sulfate chains enriched in chondroitin 6-sulfate. These proteochondroitin sulfate proteoglycans appear to contain large numbers of O-linked oligosaccharides and although the proteoglycan synthesized by arterial smooth muscle cells may have a protein core that differs from that of the cartilage monomer, it does have a hyaluronic acid binding region (Chang et al., 1983). As aggregates in the ground substance these proteoglycans undoubtedly endow tissues with viscoelasticity, but the difference in their glycosaminoglycan size and content may give these aggregates different physical properties from those in hyaline cartilage. Similar, smaller, proteoglycans having lower buoyant densities have been isolated from brain (Kiang et al., 1981) and the soft mesenchymal tissue surrounding the mineralized matrix of bone (Fisher et al., 1981). Although antisera to the latter molecule crossreact with the cartilage-specific proteoglycan, its ability to aggregate with hyaluronic acid is unknown. Large proteoglycans, that possess

low levels of L-iduronic acid residues (10%) in their dermatan sulfate side chains, have been isolated from cultured glial and glioma cells (Norling et al., 1983), bovine aorta (Oegema et al., 1979), dedifferentiated chondrocytes (Oegema et al., 1981) and embryonic skin fibroblasts (Cöster et al., 1979). They resemble the large proteochondroitin sulfate proteoglycans in size, protein content and the ability to form aggregates with hyaluronic acid. The difference in their glycosaminoglycan side chains may affect the properties of the aggregates that these proteoglycans form.

A number of other L-iduronic acid-poor dermatan sulfate proteoglycans have been isolated. Those shown not to aggregate with hyaluronic acid include a very large proteoglycan isolated from porcine follicular fluid (Yanagishita and Hascall, 1979a) and the culture medium of rat ovarian granulosa cells (Yanagishita and Hascall, 1979b) as well as smaller forms isolated from cartilage (Shinomura et al., 1983; Noro et al., 1983) and human (Salisbury et al., 1981) and pigeon (Wagner et al., 1983) aorta. The smaller L-iduronic acid-poor dermatan sulfate proteoglycans from bovine aorta (Eisenstein et al., 1975; Kapoor et al., 1981) have not been tested for their ability to aggregate with hyaluronic acid nor have similar proteoglycans from colon (Thornton and Hunt, 1984), periodontal ligament PG1 (Pearson and Gibson, 1982), sclera PG1 (Cöster and Fransson, 1981), fetal membranes (Brennan et al., 1984) and the culture medium of vascular endothelial cells (Oohira et al., 1983), arterial smooth muscle cells (Carrino and Caplan, 1984), rat ovarian granulosa cells (Yanagishita and Hascall, 1984) and corneal stroma (Cöster et al., 1983). Although all of these proteoglycans are polydisperse and appear to contain a relatively small number of long (Mr = 40,000) dermatan sulfate side chains, evidence based largely on the size of their protein cores strongly suggests that this group consists of at least several distinct proteoglycans. Although one has been proposed to be located pericellularly (Yanagishita and Hascall, 1984), the function of these proteoglycans remains somewhat obscure. It has been demonstrated that the large scleral proteoglycan (i.e., PG1) has a pronounced tendency to self-associate via its L-iduronic acid-poor dermatan sulfate side

chains (Sheehan et al., 1981; Fransson et al., 1982) and it may be possible that all of these proteoglycans are able to form self-associated aggregates at cell surfaces and/or in the extracellular matrix without the aid of hyaluronic acid. The physical properties of such aggregates are unknown but it has been suggested (Kapoor et al., 1981) that their sensitivity to shear forces may allow the aggregates to separate in tissues (such as aorta) as the pressure increases.

Smaller heparan sulfate containing proteoglycans are located pericellularly (Oldberg et al., 1979; Carlstedt et al., 1983; Rapraeger and Bernfield, 1983; Robinson et al., 1984; Yanagishita and Hascall, 1983) and in basement membranes (Hassell et al., 1980; Robinson et al., 1983; Fujiwara et al., 1984) and contain low levels of L-iduronic acid residues in their heparan sulfate side chains. Like the L-iduronic acid-poor proteodermatan sulfate proteoglycans, these proteoglycans are believed to self-associate via their heparan sulfate side chains (Fransson et al., 1981; Fransson et al, 1983) although disulfide bonded aggregates may also occur (Cöster et al., 1983; Lowe-Krentz and Keller, 1984). These proteoglycans are thought to play an integral role at the cell surface in cell-cell (Oldberg et al., 1979) and cell-substratum (Lark and Culp, 1984a; 1984b) interactions. These proteoglycans also control the charge-selective properties in basement membranes and appear to bind to native type I collagen fibrils via their glycosaminoglycan side chains (Koda and Bernfield, 1984)

Small proteochondroitin sulfate proteoglycans, apparently incapable of forming aggregates with hyaluronic acid have been isolated from bovine nasal cartilage (Heinegård et al., 1981) bone matrix (Fisher et al., 1983; Prince et al., 1983; Franzén and Heinegård, 1984), platelets (Huang et al., 1982) and concanavalin A-stimulated lymphocytes (Levitt and Ho., 1983). These molecules have small protein cores ($Mr < 46,000$) and only a few chondroitin sulfate side chains. Although the bone and cartilage proteoglycans may be related (Franzen and Heinegård, 1984), the others may not be; nor is it understood how any of these molecules function.

A small proteokeratan sulfate proteoglycan has been isolated from corneal stroma (Hassell et al, 1979; Cöster et al., 1983; Axelsson and Malmström, 1978) and although it has only 1-2 keratan sulfate side chains it has been suggested that these proteoglycans serve as hydrated "spacers" between the regularly arranged collagen fibrils in the corneal stroma ensuring its transparency (Axelsson, 1984).

A number of L-iduronic acid-rich dermatan sulfate proteoglycans have been isolated and characterized and all are small molecules with only one or a few glycosaminoglycan side chains of intermediate length and several oligosaccharides (Shinkai et al., 1983) attached to a protein core with an Mr=44,000-55,000. It is apparent that these proteoglycans represent a family of molecules that are very much alike (see Table 3) and share an amino acid composition rich in Asx, Glx and Leu (see Table 4). The reported differences in their protein content and the size of their glycosaminoglycan side chains and protein cores after digestion with chondroitinase ABC (i.e., their (ABC)cores) may be due to species and tissue differences in carbohydrate content as well as differences in analytical methodology. This species of proteoglycan (hereinafter referred to as proteodermatan sulfate; PDS) is predominant in fibrous connective tissue matrices (Table 3) but is also found in bovine aorta (Figura et al., 1975; Kapoor et al., 1981), fibroblast culture medium (Cöster et al., 1979; Carlstedt et al., 1981), bovine liver capsule (Jansson et al., 1975) and many other tissues as well. Unlike the other proteoglycans which are believed to function pericellularly and/or in the interfibrillar spaces, or whose function remains unknown, there is considerable evidence to suggest that PDS functions in tissues in close association with type I collagen fibrils.

TABLE 3
CHEMICAL CHARACTERISTICS OF L-IDURONIC ACID-RICH PROTEODERMATAN SULFATE PREPARATIONS

SOURCE	PROTEIN CONTENT	PDS MOL.WT.	(ABC)CORE[11] MOL.WT.	% L-IDUA in GAG	DS MOL.WT.	OLIGO-SACCHARIDES	REFERENCE
bovine heart valves	50%	~150,000[1]	ND	ND	ND	ND	Toole & Lowther, 1968
bovine tendon	50%	120,000[1]	ND	80%	ND	+	Anderson, 1975
bovine sclera	59%	85,000[1]	46,000[1]	50%[3]	ND	+	Coster & Fransson, 1981
calf skin	--	115,000[2]	56,000[2]	high[7]	17,000[4]	+	Fujii & Nagai, 1981
calf skin	--	120,000[2]	55,000[2]	74%[3]	17,900[4]	+	Nakamura et al., 1983
bovine skin	60%	90,000[2]	ND	75%[3]	16,500[4]	+	Pearson & Gibson, 1982
bovine perio. ligament	48%	120,000[2]	ND	75%[3]	29,000[4]	+	Pearson & Gibson, 1982
pig skin	58%	2,900,000[5]	ND	92%[3]	15,000[4]	+	Obrink, 1972
pig skin	62%	70,000[1]	44,000[2]	85%[3]	26,000[4]	+	Damle et al., 1982
Rat skin	46%	36,000[6]	ND	75%[3]	23,000[6]	+	Miyamoto & Nagase, 1980
Human cervix	35%	106,000[2]	47,000[2]	50%[10]	25,000[6]	+	Uldbjerg et al., 1983

Values determined by:
[1]Sedimentation equilibrium. [2] SDS-polyacrylamide gel electrophoresis
[3] The ratio of uronic acid liberated by chondroitinase AC II to total uronic acid liberated by chondroitinase ABC
[4] Method of Wasteson (1971) using DS-peptides produced by papain digestion
[5] End-labeling method of Hopwood & Robinson (1973). [6] Light scattering. [7] The carbazole/orcinol ratio.
[8] Method of Wasteson (1971) using DS liberated by alkali treatment
[9] Method of Wasteson (1971) using intact PDS
[10] By selective periodate oxidation of unsubstituted L-iduronic acid residues (Fransson, 1974)
[11] (ABC)core refers to the protein core produced after digestion of the proteoglycan with chondroitinase ABC.

TABLE 4
AMINO ACID COMPOSITION OF PROTEODERMATAN SULFATE PREPARATIONS (RESIDUES/1000)

	Bovine Heart Valves[1]	Bovine Tendon[1]	Bovine Sclera[2]	Calf Skin[4]	Calf Skin[5]	Bovine Skin[6]	Bovine Perio. Ligament[6]	Pig Skin[7]	Pig Skin[8]	Rat Skin[9]	Human Cervix[10]
Asx	117	110	123	113	130	122	125	134	128	152	130
Thr	57	43	49	37	42	38	39	46	42	45	45
Ser	72	79	68	62	67	62	74	69	65	64	79
Glx	140	104	122	109	100	109	108	110	104	111	110
Pro	74	87	74	92	77	70	67	79	72	81	77
Gly	95	89	84	108	72	74	80	81	76	63	85
Ala	75	53	54	51	53	50	49	53	49	46	53
Val	49	58	59	56	58	62	58	56	66	44	50
Met	4	10	7	11	12	9	9	14	6	21	3
Ileu	35	54	55	56	63	65	57	56	56	61	43
Leu	96	119	115	110	121	124	123	132	127	116	122
Tyr	20	22	15	19	28	29	29	24	23	31	20
Phe	35	28	34	40	33	34	33	34	29	27	33
Lys	50[11]	86	96	74	77	83	75	29[11]	75	63	68
His	20	29	25	24	28	28	27	22	23	20	25
Arg	49	29	32	26	30	28	31	38	32	31	41
Cys	ND[12]	ND	9	12	9	13	16	ND	15	24	17

[1]Toole & Lowther, 1968
[2]Anderson, 1975
[3]Coster and Fransson, 1981
[4]Fujii and Nagai, 1981
[5]Nakamura, Matsunaga and Shinkai, 1983
[6]Pearson and Gibson, 1982
[7]Obrink, 1972
[8]Damle, Coster and Gregory, 1982
[9]Miyamoto and Nagase, 1980
[10]Uldbjerg, Malmstrom, Ekman, Sheehan, Ulmsten and Wingerup, 1983
[11]The lysine content of these preparations is probably too low due to the likelihood of carbamylation of the free amino groups of lysine using hot 6M urea extraction methods.
[12]Not determined

1.4 Evidence for an Interaction Between L-Iduronic Acid-Rich Proteodermatan Sulfate and Collagen

The possibility that a functional association existed between PDS and type I collagen fibrils became evident when it was discovered that L-iduronic acid-rich dermatan sulfate was the most abundant glycosaminoglycan present in mature dermis (Meyer and Chaffee, 1941; Hoffman et al., 1957) and preponderated in such fibrous tissues as tendon, ligamentum nuchae and heart valves (Meyer et al., 1956). More recently, the location of this glycosaminoglycan in the body has been shown to correlate strongly with the presence of type I, and to some extent type III, collagen (Junqueira et al., 1981). Pathologically, where there is an increase in type I collagen associated with dermal scarring, corneal scarring (Anseth and Fransson, 1969), fibrosis of the lung (Motomiya et al., 1975) and in the late fibrotic stage of scleroderma (Ishikawa and Horuchi, 1975), there is also an increase in L-iduronic acid-rich dermatan sulfate.

This association is also evident from the numerous studies demonstrating that during the development of those tissues ultimately rich in type I collagen, L-iduronic acid-rich dermatan sulfate increases in the tissue as the collagen content increases and the tissue matures. Hoffman et al. (1957) originally noted that while adult pigskin contained largely dermatan sulfate and hyaluronic acid in the ratio 1.3:1, the ratio in embryonic skin was 0.2:1. Loewi and Meyer (1958) found that maturation of embryonic pigskin involved a decline in chondroitin 6-sulfate and a rise in dermatan sulfate to the extent that in the adult, dermatan sulfate represented 64% of the total glycosaminoglycan present and chondroitin sulfate was almost nonexistent. Breen et al. (1970) also found that skin from a 3 month-old fetus contained only hyaluronic acid and chondroitin 4(6)-sulfate but that by 5.5 months of age, dermatan sulfate was detectable. Horowitz and Crystal (1975) showed that fetal rabbit lungs contained chondroitin sulfate, but that with the increase in collagen content, there was also an increase in dermatan sulfate. Scott et al. (1981) found that chondroitin sulfate and hyaluronic acid preponderated in fetal rat tail tendon, while dermatan sulfate was the main glycosaminoglycan in the mature tendon. The change in glycosaminoglycan content was also related to the increase in collagen fibril diameter

(Scott et al., 1981). Kawamoto and Nagai (1976) assayed the glycosaminoglycan content of embryonic chick skin at various stages and found that at day 12 hyaluronic acid and chondroitin sulfate were the major components, but prior to the increase in collagen content on day 14, chondroitin sulfate decreased dramatically and dermatan sulfate increased sharply and continued to rise with increased collagen deposition. Nakamura and Nagai (1980), employing ^{3}H-glucosamine and ^{3}H-proline, also monitored the changes in the synthesis of the glycosaminoglycans and collagen in embryonic chick skin. They found that the synthesis of all glycosaminoglycans increased as collagen synthesis increased, but deposition of hyaluronic acid and chondroitin sulfate in the tissues decreased while deposition of dermatan sulfate increased concomitantly with the collagen. Although it was proposed early (Hoffman et al., 1957) that L-iduronic acid-rich dermatan sulfate was in some way associated with the formation of large type I collagen fibers, Parry et al. (1981), based on the accumulated data in the literature, proposed that collagen fibril diameter increased beyond 150 nm only when the relative proportions of hyaluronic acid and chondroitin sulfate declined and L-iduronic acid-rich dermatan sulfate became the predominant glycosaminoglycan in the tissue. The same hypothesis has been put forward by Scott et al. (1981), but how the change in glycosaminoglycans influences or regulates collagen fibril diameter remains obscure.

Evidence to suggest that a specific interaction may occur *in vivo* between L-iduronic acid-rich dermatan sulfate and collagen has been provided by various studies *in vitro*. In a series of experiments that physically measured the interaction between the various glycosaminoglycans and homopolymers of poly L-lysine and poly L-arginine, Gelman and Blackwell (Gelman et al., 1972; Gelman et al., 1973a; 1973b; Gelman and Blackwell, 1973a; 1973b; 1973c; 1974a) found that the interactions were electrostatic in nature and heavily dependent upon the participation of the sulfate groups, and that those glycosaminoglycans rich in L-iduronic acid (i.e., dermatan sulfate and heparin) stabilized the polycationic α helices against thermal and ionic denaturation to the greatest extent. By and large, these results have been confirmed in studies measuring the interaction between the glycosaminoglycans and collagen molecules. While high molecular

weight hyaluronic acid and chondroitin sulfate chains isolated from hyaline cartilage were able to form weak, ionic complexes with native type I collagen molecules (Mathews, 1965; Obrink and Wasteson, 1971; Obrink, 1973; Obrink et al., 1975), L-iduronic acid-rich dermatan sulfate interacted with the collagen molecules with higher affinity (Obrink and Sundelöf, 1973; Conochie et al., 1975) inducing greater stabilization to thermal and ionic denaturation (Gelman and Blackwell, 1973d; 1974b). Also, Gallagher et al. (1983) demonstrated that L-iduronic acid-rich dermatan sulfate synthesized by cultured fibroblasts remains in the medium when the cells are grown on plastic, but became specifically incorporated into collagen gels when the cells are grown on that substratum.

Evidence to suggest that intact PDS interacts with collagen molecules has been obtained through turbimetric studies that measured the effect various proteoglycans, particularly PDS, had on collagen fibrillogenesis *in vitro*. From a host of early studies it became apparent that: a) both the skin and heart valve PDS and the cartilage-specific proteoglycan preparations could retard fibrillogenesis (Mathews and Decker, 1968; Lowther and Toole, 1970; Lowther, 1972; Oegema et al., 1975); b) the PDS preparations had perhaps a higher affinity for the collagen (Toole and Lowther, 1968; Obrink and Sundelöf, 1973); c) the collagen molecules had to be native (Mathews and Decker, 1968; Lowther and Toole, 1970); d) the proteoglycans had their greatest effect when intact (Toole and Lowther, 1967; Lowther and Toole, 1970; Podrazky et al., 1971; Greenwald, 1975; Oegema et al.,1975) but that e) the results were somewhat inconclusive in that the PDS preparations did not display a significantly greater effect than the cartilage-specific proteoglycan, nor was the nature of the effect fully explainable by these methods. More recently however, Vogel et al. (1984), in a turbimetric study using various collagen preparations, demonstrated that the small L-iduronic acid rich dermatan sulfate proteoglycan isolated from bovine flexor tendon decreased the rate of fibrillogenesis much more than the large proteochondroitin sulfate proteoglycan isolated from the same source, the small proteochondroitin sulfate from cartilage and the cartilage-specific monomer and that much of the effect was mediated by the protein core of this proteoglycan as its (ABC)core was as

effective an inhibitor as the intact molecule.

That a close physical association between PDS and type I collagen probably occurs *in vivo* has been demonstrated by the need to treat tissues with 3M $MgCl_2$, 8M urea or 4M guanidinium chloride in order effectively to extract PDS. This suggests that this proteoglycan is not confined to the interfibrillar spaces or merely to the surface of the collagen fibers, but is probably within the fibers; between or on the fibrils themselves.

This contention is supported to some degree by electron microscopic studies that have used multivalent cations or cationic dyes to locate proteoglycans by way of their polyanionic glycosaminoglycan side chains. Early studies using bismuth nitrate demonstrated that the glycosaminoglycans of the large cartilage-specific proteoglycan collapsed against the protein core by precipitation with the bismuth ion forming electron dense spherical particles. Such particles were found in bovine nasal cartilage, articular cartilage and nucleus pulposus in the interfibrillar spaces and on the surface of the collagen fibrils at regular intervals (Serafini-Fracassini and Smith, 1966; Smith et al., 1967; Smith and Serafini-Fracassini, 1968), usually at the *a* and b_1 bands of each D period (see Fig. 21). In rabbit corneal stroma, stained filaments (presumed to be a protein core as highlighted by its collapsed glycosaminoglycans) ran transversely between the regularly arranged collagen fibrils and associated tangentially with the fibrils at the *a* band (Smith and Frame, 1969). The nature of this "proteoglycan" was unknown. Use of ruthenium red in conjunction with tissue fixation also revealed that a polyanionic component was associated with the collagen fibrils of subcutaneous tissue (Kajikawa et al., 1970) which on preparations of rat heart valves (Nakao and Bashey, 1972) and rat tail tendon (Torp et al., 1975) were detected between bands *d-a* on positively stained collagen fibrils. Components that had biosynthetically incorporated $^{35}SO_4$ appeared as "transverse belts" located in the vicinity of the *a* band of collagen fibrils in ear cartilage (Myers, 1976). In an attempt to increase the specificity of the reaction, dyes have been used in conjunction with $MgCl_2$ concentrations that are supposed to displace the cationic dye from polycarboxylates such as hyaluronic acid but not the sulfated glycosaminoglycans (Scott, 1980). Although not

particularly effective with ruthenium red, this "critical electrolyte concentration method" has been used with Alcian blue (Ruggeri et al., 1975) to reveal polyanionic components, presumed to be sulfated glycosaminoglycans, orthogonally arranged on collagen fibrils at regular intervals. Use of the copper-containing dyes, cinchomeronic acid and quinolinic acid, in conjunction with glutaraldehyde fixation and 0.1M-0.3M $MgCl_2$, also revealed regular, orthogonal perifibrillar arrays of a filamentous material in association with rat tail tendon collagen fibrils (Scott, 1980). The filaments appeared to form complete rings around each fibril, that were separated by the collagen band repeat distance, D, and were connected by so-called "vertical components" that ran axially between the encircling hoops. None of the filamentous material was observed inside fibrils cut in cross-section. The filaments were interpreted to be proteoglycan protein cores highlighted by the collapse of their glycosaminoglycan side chains against them. In the native tissue, the glycosaminoglycan side chains were thought to be oriented parallel to the long axis of the collagen fibril, across the various bands of a D period (Scott, 1980). In conjunction with uranyl acetate staining, the ring-like filaments were shown to be associated predominantly with the *d* band, in the gap region of the collagen fibrils (Scott and Orford, 1981). Based on the dermatan sulfate/hydroxyproline ratios and the ratio of the total length of stained filaments to collagen fibril cross-sectional area in a prescribed area of electronphotomicrographs obtained from maturing rat tail tendons, the stained components of the orthogonal array were considered to be largely dermatan sulfate and the proteoglycan, PDS (Scott et al., 1981; Scott and Orford, 1981).

1.5 Immunological Analysis of Proteoglycans

Antibodies have been used extensively as probes to study the structural and functional characteristics of connective tissue macromolecules (Furthmayr, 1982) by a number of immunological methods. Immunologic analyses of proteoglycans, however, have focused almost exclusively on the high molecular weight monomer found in cartilage. Early immunologic analyses revealed that hyaluronic acid on its own or coupled to carrier proteins was nonantigenic

in rabbits (Humphrey, 1943; Quinn and Singh, 1957). Protein-free chondroitin sulfate glycosaminoglycans from rabbit cartilage were nonimmunogenic in the species of origin (Boake and [redacted] and those from human costal cartilage were also poor immunogens in rabbits (Quinn and Ceroni, 1957) although bovine chondroitin sulfate could induce a variable response in rodents (Saunders et al., 1962). The apparent inconsistency in the immunogenicity of the glycosaminoglycans may be due to the tendency of different animal species to respond differently to large carbohydrates (Maurer and Mansmann, 1958). When a cartilage protein-polysaccharide complex (Malawista and Schubert, 1958) from bovine or human cartilage was used as the immunogen, most species developed a significant humoral response, that was based largely on the protein moiety (DiFerrante, 1963; White et al., 1963). Species specific and species common determinants were subsequently described (Loewi, 1964; Loewi and Muir, 1965) although with improvements in the preparation of the proteoglycan (Sajdera and Hascall, 1969), it became apparent that the bulk of the species specific determinants resided on the link proteins and the species common determinants were on the proteoglycan (Tsiganos, 1971) located within a highly conserved decapeptide situated between the attachment sites of adjacent glycosaminoglycan side chains (Baxter and Muir, 1972). Although it had been proposed that the link proteins were immunologically and structurally related to the hyaluronic acid binding region (HABR) of the proteoglycan (Keiser, 1975), use of more highly purified antigen preparations disproved this (Weislander and Heinegård, 1979; Poole et al., 1980a).

Antibodies specific for the proteoglycan have been used to locate the molecule in bovine articular cartilage at the light (Poole et al., 1980b) and electron microscopic levels (Poole et al., 1982), to immunoprecipitate the intracellular biosynthetic precursor of the proteoglycan protein core (Kimura et al., 1981; Geetha-Habib et al., 1984; Fellini et al., 1984), to reveal structural differences in the aggregating and non-aggregating proteoglycans in cartilage (Kimata et al., 1982b) and in a radioimmunoassay to demonstrate that the proteoglycans synthesized by presumptive embryonic chick limb bud chondroblasts are structurally different from the cartilage-specific proteoglycan synthesized by the definitive chondroblasts (Ho et al., 1977). By

selective proteolytic cleavage of the proteoglycan it has been possible to isolate different structural regions of the protein core and use them to produce antisera specific for those regions. Antibodies to the HABR and the CS-peptides were used by Weislander and Heinegård (1980) in a radioimmunoassay to demonstrate that proteoglycan preparations fractionated by size differed in the amount of CS-peptide present, as had been observed electron microscopically (Heinegård et al., 1978) and chemically (Fellini et al., 1981). These antisera have also been used to detect structurally related molecules in the other cartilages of the (bovine) body as well as in other species (Weislander and Heinegård, 1981) and to reveal that the proteoglycans synthesized by cultured glial cells (Norling et al., 1984) and the large proteochondroitin sulfate proteoglycan in bone (Fisher et al., 1983) are structurally related to the cartilage monomer. Antibodies to the HABR have also been employed in an ELISA inhibition assay to reveal possible conformational changes in the link proteins and the HABR during their interaction (Thonar et al., 1982) and to prove that the HABR is located at one end of the proteoglycan only (Buckwalter et al., 1982).

Antisera have also been raised against the basement membrane heparan sulfate proteoglycan (Hassell et al., 1980) and have been used in immunofluorescence studies (Oohira et al., 1983) and to reveal that the high and low buoyant density heparan sulfate proteoglycans share a similar protein core (Fujiwara et al., 1984). Antibodies have been raised to PG-Lt (very low buoyant density) and PG-Lb (low buoyant density) proteoglycans isolated from hyaline cartilage and have been used to show that not only do these molecules differ structurally from one another but that they differ structurally from the high buoyant density cartilage monomer as well (Shinomura et al., 1983; Noro et al., 1983). They have also been used to demonstrate that each is located in a different region of the cartilage matrix (Shinomura et al., 1984). Antibodies have been raised against the small proteochondroitin sulfate in bone and have been used to show that it is structurally related to the small proteochondroitin sulfate in cartilage (Franzen and Heinegård, 1984) but distinct from the large proteochondroitin sulfate in bone and a number of other proteoglycans (Fisher et al., 1983). Monospecific antibodies to a chondroitin sulfate proteoglycan in brain have been used to immunohistochemically locate the

proteoglycan in adult (Aquino et al., 1984a) and in developing (Aquino et al., 1984b) nervous tissue at the ultrastructural level.

These antisera have proved to be very useful adjuncts to the study of proteoglycans. However, with the advent of hybridoma methodology (Köhler and Milstein, 1975), the use of monoclonal antibodies (MAbs) to study complex macromolecules has become popular. Unlike antisera, which contain a substantial number of polyclonal antibodies (derived from different clones of plasma cells), culture fluid from an individual clone contains only one antibody that recognizes (almost always) only one determinant on the antigen. With monoclonal antibody preparations, single determinants (epitopes) may be examined independently thereby increasing the resolution of analyses even further.

Monoclonal antibodies are produced by the fusion of differentiating B cells or plasma cells with suitably mutated, nonsecreting plasmacytoma cells to yield hybrid cells that retain the ability to secrete antigen-specific antibody and the capacity to divide. As the fusion of cells occurs in a batchwise process, monoclonal antibodies are only available after a single cell, secreting an appropriate antibody, has been isolated and allowed to grow and divide as a clone. In addition to the increased resolution possible, monoclonal antibodies offer several distinct advantages over affinity purified antisera. There is less possibility of crossreaction with unrelated molecules, they may be produced by immunization with impure antigen preparations, their specificity and class does not change, large quantities of a uniform antibody preparation can be produced and, because the hybrid cells can be stored frozen, an unlimited supply is potentially available.

Monoclonal antibodies have been prepared against the cartilage proteoglycan monomer but, in each case, enzymatically treated proteoglycans have been used as the immunogen, and have resulted more often than not in the production of MAbs that do not recognize the intact proteoglycan but artifactual determinants caused by the enzyme treatment. Dorfman et al. (1980) used a hyaluronidase-treated proteoglycan preparation and while one MAb recognized the intact proteoglycan and its cell-free translation product and crossreacted with the

non-cartilaginous proteochondroitin sulfate from embryonic chick calvaria (Sugahara et al., 1980), another recognized chondroitin 6-sulfate containing tetra- and hexasaccharides with a D-glucuronic acid residue at the nonreducing terminal produced by hyaluronidase (Jenkins and Dorfman, 1981). Caterson and coworkers, using a chondroitinase ABC-digested proteoglycan preparation as immunogen, produced a rabbit antiserum and several monoclonal antibodies (Christner et al., 1980; Caterson et al., 1982; Caterson et al., 1985) shown to recognize the unsaturated derivative of the D-glucuronic acid residue remaining at the nonreducing terminal of the chondroitin 4-sulfate and chondroitin 6-sulfate chains after enzyme treatment. These antibodies have been used to study chondroitin sulfate proteoglycan biosynthesis in chicken chondrocyte cultures (Vertel and Barkman, 1984) and to detect chondroitin sulfate and dermatan sulfate proteoglycans in various tissues after treatment with chondroitinase ABC (Caterson et al., 1982; Couchman et al., 1984). Other monoclonal antibodies recognized the keratan sulfate chains themselves (Caterson et al., 1983). Monoclonal antibodies have also been produced that recognize keratan sulfate side chains using the corneal proteokeratan sulfate as immunogen (Funderburgh et al., 1982).

1.6 Objective of Project

As stated previously, an understanding of how connective tissue matrices function must come from a more complete understanding of their macromolecular components and how they interact with one another. While it is apparent that a large number of components are present in the various connective tissues and that they interact to maintain the structural integrity of these tissues, the role of PDS is likely to be somewhat different in that it appears to help regulate collagen fibril size and/or its interaction with other components. It may be one of the strict biological controls over collagen self-assembly. The most convincing evidence that PDS, among the proteoglycans, specifically interacts with type I collagen has been provided by the fibrillogenesis studies of Vogel et al. (1984), although it is not known where PDS binds to the collagen molecules to induce their effect, nor whether these "fibrils" that form are structurally

normal. The manner in which PDS might interact with type I collagen fibrils *in vivo* has been investigated in the ultrastructural localization studies of Scott and coworkers (1980; 1981). If the assumptions in these latter studies are correct, they strongly reinforce the work of Vogel et al. (1984). However, the serious drawback with the electron microscopic studies is the lack of specificity of the dye for L-iduronic acid-rich dermatan sulfate (or possibly even glycosaminoglycans) even in the presence of a "critical electrolyte concentration". Scott (1980) states that the use of cinchomeronic acid with 0.3M $MgCl_2$ can provide a high level of specificity for galactosaminoglycans, but in much of his work relies upon quinolinic acid (stated as being more prone to bind to ribonucleic acid) in conjunction with 0.1M $MgCl_2$, a concentration of salt that enhances nonspecific binding by the dye. Of particular concern was the use of quinolinic acid and low $MgCl_2$ concentrations to derive the results with developing rat tail tendon samples (Scott et al., 1981), some of which contained high levels of chondroitin sulfate which would have bound the dye. With these inherent problems there was a need to fall back on biochemical data to infer that the stained filaments were most likely to be PDS. However, there was no guarantee that the stained filaments were all proteoglycans. Another point of contention is the interpretation that the dye has consistently caused the glycosaminoglycan side chains to collapse (by precipitation) against the protein core, thus highlighting it. This may not be the case, particularly with the so-called "vertical components" of the orthogonal array. What is required is a means specifically to locate PDS in relation to native type I collagen fibrils to determine whether the protein core of PDS is indeed located in the vicinity of the d band (in the gap region) of each D period.

As has been established, largely with the cartilage-specific proteoglycan, immunological methods can be a powerful tool with which to study these structurally complex molecules. With monoclonal antibodies it should be possible to probe the structure, function and location of a proteoglycan on a determinant by determinant basis, ensuring high resolution. However, monoclonal antibodies directed against the glycosaminoglycan side chains, or artifactual determinants derived from them, cannot distinguish between 2 very different proteoglycans if

they share the same glycosaminoglycan side chains. Greater specificity, and ultimate usefulness, can be achieved with monoclonal antibodies directed against determinants on the protein core of a proteoglycan and they should be produced with this objective in mind.

Therefore, the objective of this project was to produce and characterize monoclonal antibodies to the protein core of PDS and to use them to study its structure, its relatedness to other proteoglycans and its interaction with type I collagen fibrils at the ultrastructural level.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals and Cell Lines

Four to five week old male SJL/J (H-2s) and A.CA/Sn (H-2f) mice were purchased from Jackson Laboratories, Bar Harbor, Maine while male CBA/CaJ (H-2k) and C3H.SW/Sn (H-2b) and female Balb/cCr mice of the same age were purchased from the MSB Animal Unit, University of Alberta. SJL/J and Balb/cCr mice were bred to produce SJL/JxBalb/cCr offspring used in ascites production. Older male Balb/cCr mice were also purchased locally and used as a source of normal mouse serum. The animals were housed in the Surgical-Medical Research Animal Centre and fed *ad libitum*.

The plasmacytoma cell line, 315.43, a mutant cell line derived from the Balb/c plasmacytoma MOPC 315, was generously provided by Dr. Tim Mosmann, DNAX Research Institute, Palo Alto, California. These cells are resistant to ouabain and lack the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and the ability to secrete an immunoglobulin (Mosmann et al., 1979). Human Gin-1 (apparently normal) gingiva fibroblasts (CRL #1292), human fetal skin fibroblasts (CRL #1510) and human adult skin fibroblasts (CRL #1224) were purchased from the American Type Culture Collection, Rockville, Md.

2.1.2 Hybridoma Production

RPMI 1640 powdered medium, fetal calf serum (FCS; heat inactivated) and NUNC cryotubes were purchased from GIBCO Canada, Burlington, Ont. Sterile 24 and 96 well flat bottom Linbro® tissue culture plates were purchased from Flow Laboratories, Mississauga, Ont. Hypoxanthine, thymidine, (+)-amethopterin (methotrexate), ouabain octahydrate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO)

and 2-mercaptoethanol were purchased from Sigma Chemical Co., St. Louis, Mo. Gentamycin was purchased from John's Scientific, Toronto; Ont. and 2,6,10,14-tetramethylpentadecane (pristane) from Aldrich Chemical Co.(Canada), Montreal, Que. Sterivex®-GS filters (0.22 μm) and accompanying peristaltic pump and Millex®-GS filters (0.22 μm) were purchased from Millipore Ltd., Mississauga, Ont.

Items purchased from suppliers in Edmonton were: polyethylene glycol 1450 (Baker® Grade), Freund's complete and incomplete adjuvants (Difco), sterile 100 x 20 mm and 60 x 15 mm plastic Falcon® Petri dishes, sterile 1, 2, 5 and 10 ml borosilicate serological pipets, pipet wrap, autoclavable garbage bags, autoclave tape, adsorbent cotton, hemocytometer and Fyrite® gas analyzer from Canlab; Monoject® insulin syringes from Stevens Alberta Co., Ltd.; Propper® sterilization bags (Propper Mfg. Co., Long Island City, NY) and Tower wrap from Prairie Medical Ltd.; sterile 15 ml and 50 ml Corning® screw cap centrifuge tubes and sterile tuberculin syringes and needles from Fisher Scientific; a Forma Scientific model 3029 CO_2 incubator from Caltec Scientific Ltd. and Union Carbide LRS 19 and XR 12 liquid nitrogen refrigerators and accessories from Medigas Alberta, Ltd. Purchased from sources at the University of Alberta were: methoxyflurane (Metofane®, Pitman-Moore, Inc., Washington Crossing, NJ) from the Surgical-Medical Research Animal Centre and Wescodyne® (West Chemical Products of Canada Ltd., Montreal, Que.), used as a general purpose disinfectant, from Biochemistry Stores. Alkyldimethylbenzyl ammonium chloride (Roccal®), used as a disinfectant in the incubator water supply (10 ml/5 l H_2O), was purchased from Canlab.

2.1.3 Enzyme Linked Immunosorbent Assay

Dynatech Immulon® 1 flat bottom and Cooke Microtiter® round bottom polystyrene plates, polyoxyethylene sorbitan monolaurate (Tween® 20) and H_2O_2, 30% were obtained from Fisher Scientific, Edmonton. 1,2-phenylenediamine dihydrochloride was obtained from Aldrich Chemical Co. The Titertek® Multiskan and interference filters and the 12-channel, 50-200 μl Multipipettors were purchased from Flow Laboratories, Ltd.

2.1.4 Antisera, Immunoglobulins and Serum

Peroxidase conjugated rabbit anti-mouse IgG, heavy and light chain specific (H&L), goat anti-rabbit IgG (H&L) and rabbit anti-goat IgG (H&L) antisera were purchased from Cappel Laboratories, West Chester, Pa. Peroxidase conjugated goat anti-mouse IgG (γ-chain specific), IgA (α-chain specific) and IgM (μ-chain specific) antisera, goat anti-mouse IgG $F(ab')_2$ fragments and ferritin conjugated goat anti-mouse IgG (H&L) antiserum were also obtained from Cappel. Rabbit anti-mouse immunoglobulin antiserum (DAKO) was purchased from Cedarlane Laboratories, Hornby, Ont. Mouse peroxidase anti-peroxidase was from Sternberger-Meyer Immunocytochemicals, Jarretsville, Md. Goat anti-mouse IgG1, IgG2a and IgG2b specific antisera were purchased from Meloy Laboratories, Rexdale, Ont. while rabbit anti-mouse IgG3 specific antiserum was purchased from Miles Laboratories, Rexdale, Ont. Mouse IgG was purchased from Sigma Chemical Co. and normal goat serum containing 0.2% NaN_3 was purchased from ChemBioMed, University of Alberta.

2.1.5 SDS Polyacrylamide Gel Electrophoresis and Western Blotting

Acrylamide, sodium dodecyl sulfate, ammonium persulfate, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), the high and low molecular weight standards, Coomassie Blue R250, Bromophenol Blue, nitrocellulose paper, the Trans-Blot® Cell and the Protean® Gel Electrophoresis Cell and power supplies for each were obtained from Bio-Rad Canada, Mississauga, Ont. Bovine serum albumin and 3,3'-diaminobenzidine tetrahydrochloride were purchased from Sigma Chemical Co. Pyronin Y (Matheson Coleman and Bell, Cincinnati, Ohio) was purchased from Canlab. Gels were dried using a GSD-4 Gel Slab Drier (Pharmacia Canada Ltd., Dorval, Que.) connected to a lyophilizer.

2.1.6 Nitrocellulose Electrophoresis

A Gelman electrophoresis tank (Gelman Instrument Co., Ann Arbor, Mich) was used with a BioRad Model 400 Power Supply. Barbital sodium and barbital were obtained from Fisher Scientific.

2.1.7 Column Chromatography

Sephacryl® S-300, the Fast Protein Liquid Chromatography (FPLC) system and Polyanion® SI-17 μm resin were from Pharmacia Canada Ltd. The Holochrome® spectrophotometer (Gilson Medical Electronics, Middleton, Wis.) equipped with a 40 μl, 10 mm path length cuvet was used in conjunction with an Ultrorac® 7000 fraction collector (LKB, Sweden) and a Fisher Recordall® Series 5000 recorder. Guanidinium hydrochloride (GdnHCl) was purchased from Terochem Laboratories Ltd., Edmonton.

2.1.8 Histochemistry

Bovine and porcine tissue samples were obtained fresh from Gainer's Ltd., Edmonton and fresh samples of chicken skin were obtained from the Surgical-Medical Research Animal Centre. Human gingiva was obtained from the Dentistry clinic at the time of periodontal surgery and a human skin sample was obtained from a biopsy by Dr. K. Pringle, University of Alberta Hospital. The Tissue-Tek® II Cryostat and its accessories were from Canlab, Edmonton while phosphate buffered formalin, uranyl acetate and osmic acid anhydride were obtained from Fisher Scientific. Grade 1 25% aqueous glutaraldehyde was purchased from Sigma Chemical Co. Spurr's epoxy resin components, Beem (00) embedding capsules and 400 mesh copper grids were purchased from J.B. EM Services Inc., Dorval, Que. Histochemically treated tissues were examined, and photomicrographs taken, with a Wild Leitz Diavert inverted stage light microscope equipped with a Wild Photoautomat MPS 45 and a 35 mm camera (Wild Canada, Calgary) or a Philips 410 transmission electron microscope, located in the Surgical-Medical Research Institute.

2.1.9 Enzymes

Cathepsin C (EC 3.4.14.1), Sigma X, sulfate free (16 units/mg protein) from bovine spleen and papain (EC 3.4.22.2), 2x crystallized, were obtained from Sigma Chemical Co. Chondroitinase ABC, from *Proteus vulgaris*, was from Miles Laboratories and micrococcal nuclease and trypsin-TPCK (270 units/mg) (Worthington) were purchased from Millipore Ltd. Cathepsin D was isolated from bovine thymus and purified by Dr. C.H. Pearson, University of Alberta.

2.1.10 Other Chemicals, Antigens, Apparatus and Sundries

Cetylpyridinium chloride (CPC), phenylmethanesulfonyl fluoride (PMSF), D,L-cysteine HCl, iodoacetamide and tunicamycin were purchased from Sigma. Disodium ethylenediamine tetraacetate (EDTA) was from Fisher Scientific and lactalbumin hydrolysate (Bacto-Peptone, Difco) and sodium sulfite were from Canlab. N-ethylmaleimide (NEM) and cyanogen bromide (CNBr) were from Aldrich Chemical Co. Pepstatin was from Peptide Institute, Osaka, Japan and concanavalin A was purchased from Pharmacia Canada.

Insoluble bovine elastin was from Elastin Products Co., Pacific, Mo. Bovine fibronectin, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate and hyaluronic acid were purchased from Miles Laboratories.

The Micro-ProDiCon negative pressure dialysis concentrator together with 10,000 molecular weight cut-off ProDiMem dialysis membrane assemblies were purchased from Pierce Chemical Co., Rockford, Ill. Spectrapor® dialysis membrane tubing (3,500 and 6,000-8,000 molecular weight cut-off) was also purchased from Pierce. Rolls of 10,000-12,000 molecular weight cut-off dialysis tubing were purchased from Union Carbide Canada.

The water used was distilled tap water redistilled with a Corning® Megapure distillation unit and/or deionized and sterilized using a Milli-Q® system equipped with a 0.22 μm Millistak®-GS filter (Millipore, Ltd.). Other supplies and sundries used, but not listed, were purchased from Canlab or Fisher Scientific, Edmonton.

2.2 METHODS

2.2.1 Preparation of Proteodermatan Sulfate

The sample of proteodermatan sulfate used as the immunogen and as the antigen in various immunological assays throughout the early stages of the project was extracted and purified in Dr. Pearson's laboratory using previously published methods (Pearson et al., 1978; Pearson and Gibson, 1982). Approximately 500 g (wet weight) of depilated adult bovine skin was cut into small pieces and ground to a fine powder in a Wiley mill cooled with liquid N_2. In this form, the tissue was exhaustively extracted at 6°C with a 4M GdnHCl, 0.05M sodium acetate, 0.05M Tris HCl, pH 7.0 buffer (buffer A) containing 0.02% (w/v) NaN_3 and proteinase inhibitors (5mM benzamidine hydrochloride, 1mM PMSF, 10mM EDTA, 0.5mM NEM, 0.1M 6-aminohexanoic acid, 5 μg/ml pepstatin and 5 μg/ml leupeptin). The pooled extracts were concentrated and buffer A exchanged for a 7M urea, 0.15M NaCl, 0.05M Tris HCl, pH 6.5 buffer (buffer B), containing proteinase inhibitors, by dialysis and ultrafiltration in an Amicon cell using a PM-10 membrane. The concentrated sample was then applied to a 5 x 50 cm DEAE-cellulose (Whatman Ltd., Maidstone, Kent, UK) column, previously equilibrated with buffer B, and the sample components eluted from the column in a stepwise fashion adapted from the method of Antonopoulis et al., (1974). Much of the contaminating protein and hyaluronic acid was removed by exhaustive elution with buffer B, while the more negatively charged components, including the proteoglycans, were eluted with a 7M urea, 2M NaCl, 0.05M Tris HCl, pH 6.5 buffer (buffer C). The fractions containing uronic acid were pooled and the components precipitated by adding 3 volumes of cold 98% ethanol and incubating the sample overnight at 6°C. After centrifugation, the precipitate was washed several times with 75% ethanol and redissolved in aqueous 0.50M NaCl. To isolate the components containing dermatan and/or chondroitin sulfate, CPC was added to the sample to a final concentration of 0.1% (w/v). The resulting precipitate was washed with 0.50M NaCl, 0.1% CPC and then, to remove the CPC, the sample was redissolved in aqueous 1M $MgCl_2$ and reprecipitated and

washed with ethanol. It was finally dissolved in a 7M urea, 0.05M Tris HCl, pH 6.5 buffer and applied to two 2.5 x 90 cm Sepharose CL-4B (Pharmacia Canada) columns connected in series that had been previously equilibrated with the same buffer. The most retarded peak (peak 3) detected at 280 nm contained PDS and its purity was established by amino acid analysis, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analysis of the glycosaminoglycans (liberated by papain digestion) by cellulose acetate electrophoresis (Pearson and Gibson, 1982). The purified PDS preparation contained 58% protein and 10.7% uronic acid (relative to the lyophilized weight).

During the course of the project other preparations of bovine skin PDS were necessarily used. Except for minor variations in amino acid composition, the material from all of these preparations that eluted as peak 3 from the Sepharose CL-4B columns was very similar to the initial antigen. Preparations containing PDS that eluted as peak 3 from Sepharose CL-4B columns were also obtained from bovine periodontal ligament (Pearson and Gibson, 1982), bovine fetal skin, bovine gingiva, human gingiva and unerupted bovine dental pulp. These uronic acid containing fractions were designated "PG2". All of these samples had been extracted and prepurified in essentially the same manner as adult bovine skin described above. A preparation of adult human skin proteodermatan sulfate that had been extracted with hot, 6M urea (Toole and Lowther, 1968) was generously provided by Dr. M. Longas, Mount Sinai Medical Center, New York. A preparation containing its protein core, produced by treatment of the proteoglycan with 0.5N NaOH, was also supplied by Dr. Longas.

2.2.2 Preparation of Larger Proteoglycans

Bovine gingiva, dental pulp, periodontal ligament (Pearson and Gibson, 1982) and human gingiva also contained a uronic acid-containing fraction that eluted near V_o (i.e., peak 1) from the Sepharose CL-4B columns and in each case was designated "PG1".

Bovine nasal cartilage and Swarm rat chondrosarcoma proteoglycan monomer (fraction A1D1) and link protein (fraction A1D4) preparations were generously supplied by

Dr. J.R. Baker, University of Alabama in Birmingham, Birmingham, Alabama.

2.2.3 Preparation of Proteoglycan Fragments by Enzymic Methods

2.2.3.1 Digestion of Samples with Chondroitinase ABC

The protein cores of proteoglycans with only the 'stubs' of their galactosaminoglycan side chain(s) remaining were produced using chondroitinase ABC and designated the (ABC)cores. Digestions were initially performed using a combination of the methods described by Oike et al. (1980) and Heinegård and Hascall (1974). A lyophilized sample of PDS was placed in a stainless steel tube prewarmed to 37°C. The sample was dissolved by adding 250 µl of a 0.4M sodium acetate, 0.4M Tris HCl, pH 7.3 buffer, 100 µl of aqueous proteinase inhibitors (0.1M EDTA and 0.1M NEM), 50 µl of methanolic proteinase inhibitors (0.1M PMSF and 5 mg/ml pepstatin), 0.2 units (manufacturer's units) of chondroitinase ABC/mg of PDS and H_2O to total 1 ml. The digestion proceeded at 37°C for 45 minutes. The (ABC)core was then separated from the small digestion products and inhibitors by ultrafiltration and dialysis (against H_2O) in an Amicon cell, using a PM-10 membrane, and lyophilized. Alternatively, 382 mg of GdnHCl was added to the digest which was subsequently applied to a calibrated 1 x 112 cm Sephacryl S-300 column equilibrated with a 4M GdnHCl, 0.5M sodium acetate, pH 6.8 buffer. The fractions containing the (ABC)core were pooled, exhaustively dialyzed against H_2O at 6°C and lyophilized.

It became apparent during the course of a separate study that the proteinase inhibitors inhibited the chondroitinase ABC, that their omission did not result in an obvious increase in proteinase activity (as detected on immunoblots) and that the use of the stainless steel tube as a digestion vessel was impractical for small quantities of sample. The inhibitors and the use of the stainless steel tube were therefore dropped from the protocol. Thus, many tissue samples were digested in polypropylene microfuge tubes at 37°C for 1-2 hours using 0.05-0.1 unit of enzyme/mg sample in 0.1M sodium acetate, 0.1M Tris HCl,

pH 7.3 buffer. Aliquots of the digest to be immunoblotted were usually diluted with sample buffer and applied to SDS-polyacrylamide gels immediately, while those for ELISAs were used immediately or stored for several days at 6°C. Prior to ELISA inhibition assays, the samples were boiled for 5 minutes.

Concentrated cell culture medium was digested with chondroitinase ABC by adding 0.05 units of enzyme/ml medium and incubating the solution at 37°C for 1 hour. The controls consisted of an equal amount of medium incubated without enzyme.

2.2.3.2 Papain Digestion of PDS

PDS was digested with papain by incubating 1 mg in 1 ml of a 0.1M sodium acetate, pH 6.0 buffer containing 5mM EDTA, 6mM cysteine HCl and 6.2 units of papain for 24 hours at 65°C. For subsequent immunological assays, the papain was inactivated, to prevent it from digesting the monoclonal antibodies, by adding iodoacetamide (in 3-fold molar excess to cysteine HCl) and incubating the mixture overnight in the dark at room temperature. Controls containing PDS were incubated for 24 hours at 65°C in buffer *i)* containing papain that had been previously denatured by boiling for 90 min., *ii)* lacking papain, *iii)* lacking papain and the subsequent addition of iodoacetamide and *iv)* lacking papain and cysteine HCl and the addition of iodoacetamide. These digests were stored at 6°C prior to assay by ELISA inhibition.

2.2.3.3 Trypsin Digestion of PDS

PDS (60 μg protein/ml) was dissolved in 50 μl of a 1mM $CaCl_2$, 0.05M Tris HCl, pH 7.3 buffer and then 5 μg of trypsin (1.35 units) was added. The sample was incubated at 37°C for 2 hours, then boiled for 10 minutes prior to its use in immunoblotting and ELISA inhibition assays.

2.2.3.4 Cathepsin C Digestion of PDS

PDS was digested with cathepsin C using the method developed by Dr. R. Chopra in Dr. Pearson's laboratory. A buffer activator solution (pH 5.0) was placed in a

microfuge tube warmed to 37°C and consisted of the following: 60 μl of a 4% pyridine solution, 60 μl of 5% acetic acid, 48 μl of 0.1N HCl, 1.5 μl of 0.1M EDTA, 48 μl of 0.375M 2-mercaptoethanol and 118.5 μl of a 4.5 mg/ml aqueous solution of PDS. A 15 μl aliquot (0.375 units) of thawed Cathepsin C was added to the sample and, immediately after mixing, 40 μl of the digest was removed and boiled in SDS-PAGE sample buffer. The digestion then proceeded at 37°C and further 40 μl aliquots of the digest were taken after 6 and 30 minutes. Each was immediately boiled in SDS-PAGE sample buffer. The digest remaining after 30 minutes was stored at 6°C.

2.2.3.5 Cathepsin D Digestion of PDS

PDS (90.2 μg protein) was dissolved in 50 μl of a 0.05M sodium acetate-acetic acid, pH 4 buffer and 1 μl of cathepsin D solution (1.6 μg protein) was added. After the sample was incubated at 37°C for 1 hour, several microliters of 0.1N NaOH were added to neutralize the solution which was then boiled for 7 minutes. The sample was stored at 6°C prior to immunoblotting and analysis by ELISA.

2.2.3.6 Micrococcal Nuclease Digestion of PDS

A preparation of PDS was digested with micrococcal nuclease by incubating 10 μg of PDS protein in 100 μl of a 0.4M NaCl, 10mM $MgCl_2$, 0.1mM $CaCl_2$, 0.05M Tris HCl, pH 8.3 buffer containing 504 units of enzyme for 60 minutes at 37°C. The reaction was stopped by adding SDS-PAGE sample buffer and boiling the solution for 5 minutes.

2.2.4 Preparation of PDS Fragments by Chemical Methods

2.2.4.1 Cyanogen Bromide Cleavage of PDS

To produce cyanogen bromide cleavage products of PDS, 3 mg of the intact molecule, previously dissolved in 1 ml of H_2O were added to 4 ml of 98% formic acid containing 75 mg CNBr and incubated at 30°C for 4 hours with constant stirring (prior to the CNBr treatment, the PDS sample had been reduced for 22 hours under N_2 in a 0.2M

NH_4HCO_3, pH 7.2 buffer containing 25% (v/v) 2-mercaptoethanol, dialyzed exhaustively against H_2O and lyophilized). Upon adding the sample to the formic acid, some of the preparation precipitated and this insoluble fraction of the CNBr digest was later removed by centrifugation, washed several times with H_2O and lyophilized. The supernatant (i.e., the soluble fraction) was diluted with 20 volumes of H_2O and lyophilized.

To isolate the N-terminal cyanogen bromide peptide the lyophilized supernatant was dissolved in a 7M urea, 0.15M NaCl, 0.05M Tris HCl, pH 6.5 buffer and injected onto a 0.5 x 10 cm Polyanion SI-17 (FPLC) column previously equilibrated with the same buffer. The column was eluted at 0.5 ml/min with this buffer until the absorbance at 230 nm returned to baseline level. The material still bound to the resin was eluted at the same flow rate with a 60 ml linear NaCl gradient using 7M urea, 2M NaCl, 0.05M Tris HCl, pH 6.5 as the second buffer. Fractions containing dermatan sulfate (as determined by uronic acid analysis) were pooled, dialyzed against water and lyophilized. The sample was redissolved in 1 ml of a 7M urea, 0.3M NaCl, 0.05M Tris HCl, pH 7.2 buffer and applied to a 1 x 112 cm Sephacryl S-300 column. The elution of sample components was monitored at 230 nm and fractions containing uronic acid were pooled and dialyzed exhaustively against Milli-Q® water at 6°C using 3,500 molecular weight cut-off dialysis tubing and lyophilized.

2.2.4.2 β-Elimination Sulfite Addition of PDS

PDS (300 μg protein) was added to 600 μl of a fresh 0.2M sodium sulfite, pH 11.6 solution in a polypropylene microfuge tube and stirred at room temperature for 22 hours. The pH of the solution was monitored occasionally with Dual-Tint pH paper and maintained within the pH range 11-12 by the addition of several microliters of 1N NaOH. The sample was neutralized by the addition of 5 μl of 1M acetic acid and immediately diluted with sample buffer and boiled for 5 minutes prior to immunoblotting. Another preparation, that had been dialyzed by ultrafiltration in an Amicon cell (PM 10 membrane) and analyzed for carbohydrate content was generously supplied by N. Winterbottom as a standard for immunoblotting purposes.

2.2.4.3 Treatment of PDS with Anhydrous HF

PDS that had been treated with anhydrous HF for 2 hours at 0°C in an apparatus in the Department of Biochemistry was kindly provided by N. Winterbottom. The sample had then been dissolved in a 4M GdnHCl buffer, dialyzed by ultrafiltration and lyophilized. Amino acid analysis indicated that some hexosamine was still present in the preparation, but that the glucosamine and galactosamine content of the sample had been decreased by approximately 65% and 94% respectively (Pearson et al., 1983). The lyophilized sample was dissolved by boiling in SDS-PAGE sample buffer and used for immunoblotting purposes.

2.2.5 Preparation of Fibroblast Culture Medium

Medium from confluent human fibroblasts was prepared in the following way. The cells, previously grown to confluency in 5% CO_2 at 37°C in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 10% FCS and antibiotics, were washed 3 times with sterile saline and reincubated in LH medium consisting of DMEM containing 0.5% (w/v) lactalbumin hydrolysate, 0.3 μg/ml fungizone (Gibco) and 25 μg/ml concanavalin A. Conditioned LH medium was collected and fresh medium added every 24 hours for 3 days. The pooled medium was concentrated with a Micro-ProDiCon assembly and stored at 6°C. An aliquot of this medium was digested with chondroitinase ABC (as described above) and both the digested and undigested media analyzed by immunoblotting methods. In a separate experiment, confluent gingival fibroblasts were incubated in the LH medium supplemented with 50 μg/ml of tunicamycin. This medium was similarly pooled, concentrated, digested and analyzed.

Medium containing calf serum from a confluent culture of monkey (*Macaca nemestrina*) arterial smooth muscle cells was kindly provided by Dr. T.N. Wight, University of Washington, Seattle, Wash.

2.2.6 Preparation of Collagen Samples

Acid soluble type I collagen was isolated from calf skin as described by Pontz et al., (1970). Following an exhaustive extraction with 1.0M NaCl, 0.05M Tris HCl, pH 7.5 at 6°C, the finely powdered skin was extracted 3 times with a 0.1M sodium citrate, pH 3.7 buffer. After centrifugation, the solubilized collagen in each extract was precipitated by dialysis against H_2O, redissolved in dilute acetic acid, dialyzed against H_2O and lyophilized.

Type III collagen was obtained from calf skin employing the method of Chung and Miller (1974). Following exhaustive extraction with a neutral salt solution, the powdered skin sample was digested with pepsin (100 mg/g sample) in 0.5M acetic acid at 6°C for 24 hours. After centrifugation, the solubilized collagen was precipitated by adding solid NaCl to adjust the solution to 0.9M and stirring it in the cold overnight. The precipitate was redissolved in 0.5M acetic acid and dialyzed against a 1.7M NaCl, 0.05M Tris HCl, pH 7.5 buffer. The type III collagen in the precipitate was purified by repeated dissolution in 1.0M NaCl, 0.05M Tris HCl, pH 7.5 buffer and precipitation by the addition of NaCl to a final concentration of 1.7M. The sample was finally dissolved in dilute acetic acid, dialyzed against H_2O and lyophilized.

Type II collagen was isolated from bovine femoral articular cartilage by the method of Herbage and Buffevant (1974). Powdered cartilage tissue was extracted first with a neutral salt solution and then 0.2M NaOH prior to digestion with pepsin as described above. The solubilized collagen was partially purified by repeated precipitation from a 1.0M NaCl, 0.05M Tris HCl, pH 7.5 buffer with 2.5M NaCl, dialyzed against H_2O and lyophilized.

Bovine type IV collagen was extracted from anterior lens capsules by pepsin digestion (Kefalides and Denduchis, 1969) and purified by salt precipitation as described for type II collagen.

Types II, III and IV collagen were further purified by ion exchange chromatography using CM-32 carboxymethylcellulose resin (Whatman). The purity of the collagen samples was determined by amino acid analysis and SDS-polyacrylamide gel electrophoresis and compared with published data.

2.2.7 Immunogenetic Analysis

Five mice from each of the four inbred strains (SJL/J, A.CA/Sn, C3H.SW/Sn and CBA/CaJ) were anesthetized and primed with 0.1 ml of a 0.5 mg/ml aqueous solution of PDS emulsified in an equal volume of Freund's complete adjuvant via 2 subcutaneous injections in the back. The mice were boosted intraperitoneally with the same amount of PDS in Freund's incomplete adjuvant on days 10 and 20 and with approximately half the amount on day 36. Anti-PDS antiserum was obtained from 1 mouse from each group following exsanguination by cardiac puncture on days 20, 30 and 47. The antisera were titered by ELISA. Three non-immune mice from each strain provided normal sera.

2.2.8 Hybridoma Production

The methods used in the production of the hybrid cells were adapted from those used by Dr. Tim Mosmann, DNAX Research Institute, Palo Alto, Calif. Minor modifications were made, however, to try to increase the success of the fusion experiments. For example, the water used to make up the RPMI 1640 medium was first double distilled, then pumped through a Milli-Q deionizer. In addition to sodium bicarbonate, HEPES (20mM) was added to the medium when it was prepared and the pH adjusted to 6.8, as the cells tolerated a slightly acidic medium very well but were intolerant of medium at pH 7.5 or higher (which could be achieved during prolonged manipulations in the containment cabinet when buffered with bicarbonate alone). Following 0.22 μm filtration of the medium into 500 ml serum bottles, 25 ml was typically taken from each bottle and cultured in 7.5% CO_2 at 37°C in an attempt to detect any contaminating infection prior to its use. The sterile RPMI 1640 medium was stored at 6°C in the dark. It also became apparent that the particular lot of fetal calf serum (FCS) used could significantly influence the results. Therefore, a preliminary fusion experiment was carried out with spleen cells from 2 Balb/cCr mice immunized with sheep red cells using 3 different lots of FCS. The lot of serum yielding the highest average number of hybrid cell clusters from both mice after 10-14 days in culture was selected for use in this project (Lot No. 29K9517).

The various chemicals used in the growth and selection media were prepared beforehand as concentrated stock solutions and sterilized by 0.22 μm filtration as necessary. A 50mM solution of 2-mercaptoethanol was made by diluting 351 μl in 100 ml H_2O and stored at 6°C. Ouabain (10mM) in RPMI 1640, made in 100 ml quantities, was stored at 6°C and used within 2 weeks. Aqueous hypoxanthine (10mM) and thymidine (3mM) solutions were made separately in 25 ml quantities and stored at -20°C (hypoxanthine required the addition of several drops of concentrated HCl to dissolve completely). A 25 ml aqueous solution of methotrexate (0.5mM) was made in H_2O rendered slightly alkaline by the addition of 0.1 N NaOH and stored at -20°C. Gentamycin was purchased sterile in rubber stoppered vials at a concentration of 50 mg/ml and represented a 1000-fold concentrated stock solution. It was kept at room temperature and aliquots withdrawn with insulin syringes. The 40% (w/w) polyethylene glycol (PEG) solution was made by autoclaving 40 g of PEG for 20 minutes and, once cooled to 60°C in a water bath, diluted with 60 ml of sterile RPMI 1640, thoroughly mixed and stored at 6°C.

Growth medium for the 315.43 cells consisted of RPMI 1640 containing 5% FCS, 50 μM 2-mercaptoethanol and 50 μg/ml of gentamycin. A stock of these cells was kept in several small Petri dishes cultured at 37°C in 7.5% CO_2. A viable number of healthy cells was maintained by reducing the cell population in each dish every second day and adding fresh growth medium. Several days prior to a fusion experiment, the stock cells were distributed into 3 large Petri dishes containing 25-30 ml of fresh growth medium and allowed to reach a high density timed to coincide with the fusion. All these cells, and the hybrid cells derived from them, were manipulated using Level B containment procedures in accordance with the "MRC Guidelines for Handling Recombinant DNA Molecules and Animal Cells and Viruses". All plastic and glassware used to handle such cells were autoclaved prior to disposal or washing.

2.2.8.1 Fusion Protocol

The fusion protocol was based on the method of Galfre et al., (1977) as modified by Mosmann and coworkers (Longenecker et al., 1979). Mice were immunized with PDS essentially as described above. Three days after the last booster injection, a mouse was

anesthetized and exsanguinated by cardiac puncture (the anti-PDS antiserum obtained was stored at 6°C and later titered by ELISA). After thoroughly soaking the mouse with 70% ethanol, the spleen was removed aseptically in a horizontal laminar flow hood and immersed in 10 ml of RPMI 1640 containing 20% FCS and gentamycin. Using sterile needles on syringes, spleen cells were released into the medium first by gently perfusing the spleen and later by teasing the spleen apart. Remnants of the spleen capsule and stroma were removed. The cell suspension was transferred to a 50 ml Corning tube and the larger white (spleen) cells counted with a hemocytometer. Healthy 315.43 myeloma cells, previously grown to high numbers in large Petri dishes, were pooled, counted and mixed with the spleen cells at a ratio of 1:5. After pelleting the cells at 600xg for 10 minutes and discarding the supernatant, they were washed once in 45 ml of RPMI 1640 and then gently resuspended in 2 ml of a 40% polyethylene glycol (PEG) solution. Following 2 minutes incubation with gentle agitation in a 37°C water bath, the cells in the PEG solution were diluted with 20 ml of RPMI 1640 added dropwise over 2-2.5 minutes. While the RPMI 1640 was added, the cell suspension was constantly swirled to ensure rapid mixing. After pelleting at 600xg for 10 minutes and discarding the supernatant, the cells were gently resuspended in 25 ml of medium containing 20% FCS, transferred to a large Petri dish and incubated at 37°C in 7.5% CO_2 for 1-2 hr. While they were incubating, the selection medium was made, its volume based on the desired final plasmacytoma cell concentration of 10^5 cells/ml. The selection medium consisted of growth medium containing 10% FCS supplemented with 100 μM hypoxanthine, 0.5 μM amethopterin, 30 μM thymidine, 1 mM ouabain and whole blood from Balb/cCr mice diluted to 10^7 red cells/ml. The blood had been obtained aseptically by cardiac puncture and immediately diluted into 80 ml of RPMI 1640 and counted. The 25 ml mixture of spleen, plasmacytoma and fused cells were added to the selection medium and 2.5 ml aliquots were dispensed into the wells of 24 (large) well Linbro plates and reincubated at 37°C in 7.5% CO_2.

2.2.8.2 Isolation of Clones

After 8-10 days, when growing hybrid cell clusters were macroscopically visible in a number of wells, 50 μl of medium were taken from each well of all plates and tested by ELISA for the presence of anti-PDS antibodies. A well was regarded as positive if the absorbance at 492 nm was 2-3 times that of the great majority of wells (i.e. background). Individual cell clusters in a positive well were removed with a Pasteur pipet and the cells from each cluster distributed equally into 3 wells (containing 200 μl of growth medium with 10% FCS supplemented with hypoxanthine, thymidine and blood cells) of a 96 (small) well plate. The tranferred cells were allowed to grow for 3-5 days before 50 μl of medium from each of the small wells were again tested for anti-PDS antibodies by ELISA. At this point, wells were regarded as positive if the absorbance value was 2-3 times higher than that of the original large well. The cells from one of these positive small wells were then recloned in growth medium (supplemented with whole blood) at progressively higher dilutions, the final at 0.3 cells/well. At each recloning step, medium from selected wells was tested by ELISA and a single cluster was removed from a positive well and recloned further. A single round cluster in an ELISA positive well derived from cells recloned at 0.3 cells/well was regarded as a clone.

To maximize the yield of anti-PDS hybrid clones from a single fusion experiment, medium from the original large wells was reassayed by ELISA 2-3 more times and the cell clusters from newly discovered positive wells were also advanced through the culture and recloning procedures.

2.2.8.3 Manipulation of Clones

Generally, 3 sister clones were grown to high density. The clones were initially transferred to large wells and cultured in growth medium containing blood cells, but as their numbers increased they were weaned from the blood cell supplement and transferred to small, then large, Petri dishes. While 2 of these clones were then frozen and stored, the third was continuously cultured as a source of supernatant medium containing monoclonal

antibody and as a stock line for ascites production. Supernatant medium from a clone was stored frozen; or pooled and stored in 50 ml Corning tubes at 6°C. Tubes opened for use were supplemented with NaN_3.

For ascites production, hybrid cells were pooled, counted and pelleted at 600xg for 5 minutes. The supernatant medium was kept and the cells were washed several times in 35 ml of RPMI 1640. They were then resuspended in RPMI 1640 at 2 x 10^6-10^7 cells/ml and 0.5 ml was injected intraperitoneally into pristane-primed, irradiated (500 rads) SJL/JxBalb/cCr mice. The animals were housed in filter cages in a chemical fume hood. The mice had received 0.5 ml of pristane intraperitoneally approximately 2-3 weeks earlier and had been irradiated the day before receiving the hybrid cells. When an ascites tumor became apparent, the mouse was anesthetized and the ascites fluid drained into a 15 ml Corning tube via a 16 gauge needle inserted into the lower abdomen. Fibrin was removed by centrifugation at 2000 rpm for 8-10 minutes and the ascites fluid divided into aliquots in 1.5 ml microfuge tubes and, with a few grains of NaN_3, stored at 6°C.

Cells to be frozen were pooled, counted, and after they were pelleted, resuspended at a concentration of 1-2 x 10^6 cells/ml in FCS containing 5% DMSO. The cells were then dispensed into 1.5 ml NUNC cryotubes and, after freezing overnight at -65°C, catalogued and transferred to a liquid N_2 refrigerator and stored.

2.2.9 Enzyme Linked Immunosorbent Assay

The ELISA technique used was based on the method of Rennard et al. (1980) developed for other connective tissue components. PDS was dissolved in H_2O at 1 mg/ml and 80 µl diluted in 80 ml of a 35mM $NaHCO_3$, 15mM $NaCO_3$, pH 9.2 buffer containing 0.02% NaN_3 (w/v). 200 µl of this antigen solution were distributed into each of 88 wells (200 ng/well) in Immulon 1 flat bottom polystryrene plates. Each plate was covered tightly with Parafilm® and incubated for at least 18 hours at 6°C prior to use. Typically, these plates were used several days to several weeks later.

Prior to starting an assay, antigen coated plates were warmed to room temperature and then rinsed 3 times with PBS/T (0.137M NaCl, 2.7mM KCl, 4mM Na_2HPO_4-$7H_2O$, 1.5mM KH_2PO_4, pH 7.2 containing 0.05% Tween 20 (v/v)). Each rinse lasted 3 minutes. The antigen coated wells were then incubated at room temperature in turn with: (*i*) solutions being tested for anti-PDS antibody (diluted in PBS/T) for 60-120 minutes, (*ii*) peroxidase conjugated rabbit anti-mouse IgG (H&L) antiserum (diluted 1:1000 with PBS/T) for 60 minutes and (*iii*) an aqueous solution of 0.01% o-phenylenediamine, 0.003% H_2O_2 for the time necessary to generate a suitable level of color; usually 30-60 minutes. Each of these steps was preceded by 3 rinses of the wells with PBS/T. The substrate was incubated in the dark to minimize nonspecific color production caused by light activation of H_2O_2. The enzyme reaction was terminated by adding 50 µl of 4M H_2SO_4 and the result quantitated with a Multiskan equipped with a 492 nm interference filter. For hybridoma screening purposes, 50 µl of medium was added to 150 µl of PBS/T in antigen coated wells and incubated for 120 minutes. For titration of antisera or fluids containing monoclonal antibody, serial dilutions were made in PBS/T in round bottom polystyrene plates. The solutions were subsequently transferred to the antigen coated wells with the Multipipettor and incubated for 60 minutes. Titration end points represented the point on the plotted titration curve where the absorbance was twice the control level.

Peroxidase conjugated goat anti-mouse IgG, IgA and IgM specific antisera were substituted as the secondary antiserum to determine the class of each monoclonal antibody. To determine the IgG subclass of the MAbs, goat anti-mouse IgG1, IgG2a, IgG2b or rabbit anti-mouse IgG3 specific antisera were used in conjunction with peroxidase conjugated rabbit anti-goat or goat anti-rabbit IgG antisera as the developing antibodies.

Competitive binding ELISAs were designed to reveal whether the anti-PDS monoclonal antibodies recognized different epitopes. These assays were done using Immulon plates coated with 50 ng PDS per well. Serial dilutions of each monoclonal antibody were made in PBS/T alone or in PBS/T containing the same volume of a second MAb and then transferred to antigen coated wells. The ELISA was then completed as usual. Both supernatant and ascites fluids were

used for these assays. The maximum absorbance generated by each of the 2 MAbs alone was compared with that generated by the mixture of the two antibodies. Lack of a significant increase in absorbance as a result of mixing was interpreted as competition between the respective antibodies for the same, adjacent or closely related determinants.

ELISA inhibition assays were also done essentially as described by Rennard et al., (1980). The inhibitor solution was diluted serially in round bottom microtiter plates and then the same volume of an appropriate concentration of hybridoma supernatant or ascites fluid was added to each well and the mixture incubated overnight at 6°C. Once warmed, the MAb-inhibitor solutions were transferred to washed PDS coated Immunlon 1 plates and incubated for 30 minutes at room temperature. Following three 3 minute washes with PBS/T, the wells were incubated in turn with: (*i*) rabbit anti-mouse immunoglobulin (diluted 1:1000 in PBS/T) for 30 minutes, (*ii*) peroxidase conjugated goat anti-rabbit IgG (diluted 1:1000 in PBS/T) for 30 minutes and (*iii*) substrate. The monoclonal antibody solution in PBS/T alone was used to establish the upper limit of color production; solutions lacking a specific antibody to PDS (controls) were used to set the lower limit. Normal mouse serum (1:100), supernatant from the parent plasmacytoma cell line, mouse IgG (10 μg/ml) or PBS/T alone were used as controls.

2.2.10 Immunoblotting

Antigen samples were typically dissolved in or diluted with a 0.05M phosphate sample buffer, pH 7.2 containing 1% SDS, 2.2M urea and 2-mercaptoethanol (2% or 10%) and heated to 45°C for 30 minutes or boiled for 5 minutes (Weber and Osborn, 1975). Aliquots (30-60 μl) were then applied to the appropriate lanes of an SDS polyacrylamide slab gel and electrophoresed in a 0.1% SDS, 0.05 M sodium phosphate, pH 7.2 buffer for 30 minutes at 50 volts, 36 mA and then at 100 volts, 74 mA for the duration of the run. When the electrophoresis was terminated, the Bromophenol blue dye front in each lane was marked with Pyronin Y and the gel was laid over a piece of nitrocellulose paper soaking in a 0.05 M sodium

phosphate, pH 7.2 buffer. After inserting the sample assembly into a water cooled Trans-Blot cell filled with the same buffer, the antigen samples were transferred from the gel to the nitrocellulose paper overnight using a 9 volt, 375 milliampere current. At the completion of the transfer, the gel was stained for 1 hour with Coomassie Blue R250 (Mechanic, 1979). The immunochemical staining of the nitrocellulose paper was based on the method of Towbin et al., (1979). While selected lanes of the nitrocellulose paper were stained with Amido black for 1-2 hours, the remainder were immersed in Tris buffered saline (TBS; 0.15M NaCl, 0.05M Tris HCl, pH 7.6) containing 2% (w/v) bovine serum albumin and incubated for 45 minutes at 37-40°C. The paper was then given three 5 minute washes in TBS and cut into as many strips as there were different control and MAb solutions used. Each lane per strip was incubated for 120 minutes at room temperature in 4 ml of a solution consisting of 2 ml TBS, 1.6 ml TBS/2% BSA, 0.4 ml normal goat serum and 4 µl of monoclonal ascites fluid, or 40 µl of normal Balb/cCr serum, and washed again in TBS. The strips were then incubated for 90 minutes in the same solution containing a 1:600-1:800 dilution of peroxidase conjugated rabbit anti-mouse IgG. After four 5 minute washes in TBS, the strips were incubated in 6 ml/lane of 0.05% diaminobenzidine tetrahydrochloride, 0.01% H_2O_2 in TBS for 10-15 minutes. The reaction was stopped by soaking the strips in H_2O for 20 minutes. To highlight the diaminobenzidine precipitate, the strips were soaked in a 0.01% (v/v) aqueous solution of OsO_4 for several minutes then rinsed in H_2O for 1 hr and dried. The strip of nitrocellulose paper stained with Amido Black was destained in a 7.5% aqueous solution of acetic acid, rinsed thoroughly with H_2O and dried.

2.2.11 Nitrocellulose Electrophoresis

The procedures used were essentially those employed for cellulose acetate electrophoresis in Dr. Pearson's laboratory. Samples were dissolved in a 0.06M barbital buffer, pH 8.6 and 4 µl aliquots were applied to 2.5 x 12 cm strips of nitrocellulose paper that had been soaking in the same buffer. Electrophoresis proceeded for 1 hr at room temperature using 0.8 mA per strip.

Strips were stained with toluidine blue, Amido black or fixed in 0.1% aqueous CPC for 30 minutes, rinsed in water and stained immunochemically with the MAbs as described above.

2.2.12 Light Microscopic Immunohistochemistry

Fresh tissue samples were cut into small pieces and individual pieces were either embedded in OCT Compound and frozen at -22°C in a cryostat chamber or fixed in 10% buffered formalin or 2.5% glutaraldehyde at room temperature for at least 4 days prior to embedding in paraffin. Whether frozen or fixed, 5-6 μm sections of tissue were cut and placed on glass slides. The wax embedded sections were warmed overnight to attach them securely to the slides. Other than necessarily dewaxing the paraffin embedded sections in several changes of xylene and rinsing in 98% ethanol, both frozen, unfixed and fixed sections were immunochemically stained the same way using the peroxidase anti-peroxidase method outlined by Sternberger, (1979).

Sections were first pretreated in absolute methanol containing 2% H_2O_2 (v/v) for 30 minutes, then given three 5 minute washes in TBS (0.15M NaCl, 0.05M Tris HCl, pH 7.6). Each section was then covered with 150 μl of TBS containing 3% normal goat serum (NGS) and incubated at room temperature for 30 minutes. The sections were then drained (not washed) and covered with 150 μl of TBS/1% NGS containing a suitable dilution of monoclonal antibody or a 1:100 dilution of normal Balb/cCr serum. The slides were incubated in a humidified container overnight at 6°C. On the following day, after they had warmed to room temperature and been washed in TBS, the sections were incubated successively with 150 μl of: (*i*) a 1:40 dilution of goat anti-mouse IgG F(ab')$_2$ fragments in TBS for 45 minutes, (*ii*) a 1:80 dilution of peroxidase mouse anti-peroxidase in TBS/1% NGS for 30 minutes and (*iii*) 0.05% diaminobenzidine tetrahydrochloride, 0.01% H_2O_2 in TBS for 10-15 minutes. Each step was preceded by 3 or 4 five minute washes in TBS. The enzyme reaction was stopped by immersing the slides in H_2O in a Wheaton staining dish and left under running tap water for 30-45 minutes. After dehydration in a graded series of ethanol and xylene, the sections were mounted with glass

coverslips. Some sections were counterstained with Alcian blue for 20 minutes prior to dehydration. All sections were examined with a Wild Leitz Diavert inverted stage light microscope and photomicrographs taken using the attached Photoautomat MPS 45 and 35 mm camera and Panatomic X film used at ASA 80. The film was processed and prints made by Photographic Services, Faculty of Dentistry.

2.2.13 Electron Microscopic Immunohistochemistry

All solutions used in these procedures were filtered with 0.22 μm Millex-GS filters.

2.2.13.1 Peroxidase Anti-Peroxidase Method

For peroxidase anti-peroxidase immunochemical staining, formalin fixed pieces of adult bovine tail tendon were placed in PBS in a sterile Petri dish and thoroughly teased apart to expose very thin fibers and their constituent fibrils. After placing small specimens into 1.5 ml microfuge tubes and washing each for 10 minutes in PBS three times, the samples were incubated for 30 minutes in absolute methanol containing 2% H_2O_2 and rewashed in TBS. Following a 30 minute incubation in TBS containing 20% NGS, the samples were incubated overnight at 6°C in TBS containing 10% NGS and a suitable dilution of monoclonal antibody or a 1:100 dilution of normal Balb/cCr serum. Samples were also incubated overnight in TBS containing 10% NGS alone or in TBS containing 10% NGS and monoclonal antibody that had been preadsorbed with 250 μg of PDS. On the following day the tendon samples were warmed and, after washing in TBS, incubated at room temperature with: (*i*) a 1:80 dilution of goat anti-mouse IgG $F(ab')_2$ fragments in TBS containing 10% NGS; (*ii*) a 1:300 dilution of peroxidase mouse anti-peroxidase in TBS containing 20% NGS for 30 minutes, (*iii*) 2.5% glutaraldehyde (v/v) in PBS for 60 minutes and (*iv*) substrate in TBS for 5 minutes. Each step was preceded by three 10 minute washes in buffer. The enzyme reaction was stopped by washing the samples in H_20 and the diaminobenzidine precipitate rendered electron opaque by incubating the samples on ice in 500 μl of a 0.1M cacodylate, pH 7.0 buffer containing 1.0% OsO_4 for 60 minutes. After

thoroughly washing the samples in H_2O, they were dehydrated in a graded series of ethanol followed by acetone and then left overnight in a 1:1 mixture of acetone:Spurr's resin. The next day the samples were passed through a 1:2 mixture of acetone:Spurr's resin and later in the day immersed in pure resin in Beem capsules and left under vacuum overnight. The embedded samples were cured at 70°C for 10-24 hours. Thin sections, on 400 mesh copper grids, were examined directly or stained with aqueous 5% uranyl acetate for 20 minutes and Reynold's lead citrate (1.33 g $Pb(NO_3)_2$, 1.76 g $Na_3(C_6H_5O_7).2H_2O$ and 8 ml 1N NaOH in 50 ml of freshly boiled and cooled distilled water) for 5-10 minutes prior to viewing with a Philips 410 transmission electron microscope.

2.2.13.2 Ferritin-Conjugated Antibody Method

After teasing pieces of adult bovine tail tendon apart in saline, they were treated with 2% H_2O_2 in methanol, rewashed and incubated for 30 minutes in TBS containing 30% NGS. Once drained, the samples were incubated overnight at 6°C in TBS containing 30% NGS and a suitable dilution of monoclonal antibody supernatant fluid or a 1:500 dilution of normal Balb/cCr serum. Samples were also incubated overnight in TBS containing 30% NGS. After warming, the samples were given four 10 minute washes with TBS and reincubated overnight in TBS containing 30% NGS and a 1:40 dilution of ferritin-conjugated goat anti-mouse IgG (H&L) antiserum. On the following day the samples were washed extensively (nine 10 minute washes) with TBS and then treated with aqueous 2.5% (v/v) glutaraldehyde for 45 minutes, washed with TBS and water and dehydrated with ethanol and acetone. The samples were embedded in Spurr's resin as described above. Thin sections were positively stained with uranyl acetate for 20 minutes prior to viewing with the Philips 410 transmission electron microscope. Electron photomicrographs, made from 35 mm negatives, were used to evaluate the location of the ferritin particles in relation to the collagen banding pattern.

2.2.14 Analytical Methods

2.2.14.1 Amino Acid Analysis

For amino acid analyses, between 50-100 μg (protein) of the sample was placed in an acid washed, screw capped tube with 200 μl of constant boiling 6N HCl (Pierce Chemical Co) and 8 μl of a 5% phenol solution. Following hydrolysis under N_2 for 22 hours at 110°C the HCl was evaporated by a N_2 stream. The hydrolysate was dissolved in 0.01N HCl and analyzed with a Beckman 121 MB amino acid analyzer. The calculated amino acid contents of a sample were based on a chromatograph of amino acid standards.

To determine homoserine content, the hydrolysate was incubated in 100 μl of 0.2N NaOH for 5 minutes and then neutralized with the same volume of 0.2N HCl prior to the addition of an appropriate volume of 0.01N HCl.

Hexosamine content of samples was determined with the amino acid analyzer after hydrolysis in 4N HCl under N_2 at 100°C for 18 hr.

2.2.14.2 Determination of Sample Protein Content

The protein content of a sample was determined by amino acid analysis or by the method of Lowry et al., (1951) using bovine serum albumin as the protein standard. The color produced by the Lowry method was quantitated at 760 nm using a Gilford Spectrophotometer equipped with a Rapid Sampler.

2.2.14.3 Uronic Acid Analysis

The uronic acid content of samples or 3- to 4-fold dilutions of column fractions was determined by an automated method (Rosenthal et al., 1976; Pearson and Gibson, 1982) using a Technicon autoanalyzer with m-dihydroxyphenol and H_2SO_4 as the principal reagents. Color production was quantitated at 530 nm with a Holochrome spectrophotometer.

3. RESULTS

3.1 Immunogenetic Analysis

An immunogenetic analysis of 4 inbred mouse strains was carried out to determine which strain was capable of mounting the most potent antibody response to intact L-iduronic acid-rich adult bovine skin proteodermatan sulfate. SJL/J (H-2s) mice provided the highest antibody titers after the second and third booster injections, although the response of A.CA/Sn (H-2f) mice ultimately rose to the same level (Fig. 5). The antibody response of CBA/CaJ (H-2k) mice decreased with continued boosting, while C3H.SW/Sn (H-2b) mice consistently provided the lowest antibody titers by an order of magnitude (Fig. 5).

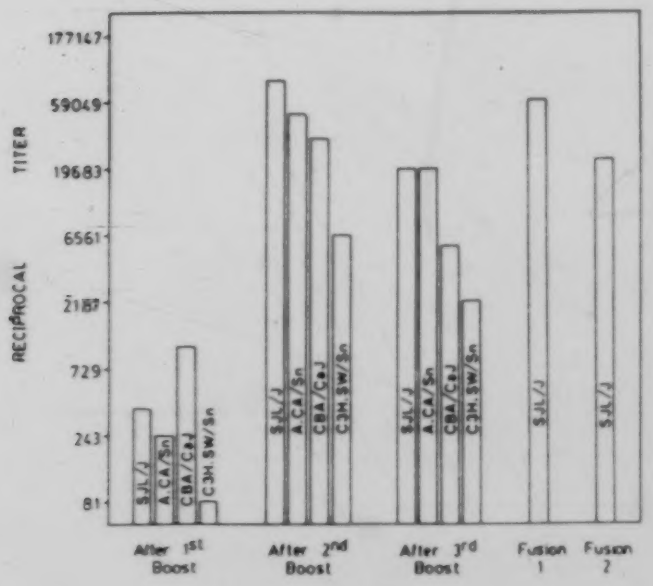

Fig. 5. Antibody response of inbred mice to bovine skin proteodermatan sulfate. Antisera were obtained from mice of each strain approximately 10 days after each booster injection and titered by ELISA using serial 3-fold dilutions. The antiserum titers of the SJL/J mice used in the fusion experiments are also indicated.

3.2 Hybridoma Production

Based on the results of the above immunogenetic analysis, 2 more SJL/J mice were primed with PDS and, after receiving 2 and 3 booster injections respectively, their spleen cells were fused with the 315.43 plasmacytoma cells. Antisera, obtained at the time of splenectomy, were subsequently titered as a check on the immunization protocol and to help verify the results (Fig. 5) of the original immunogenetic analysis .

The first fusion resulted in the formation of approximately 400 hybrid cell clusters in a total of 156 wells. Only 5 wells were shown to be positive and became so only after day 19 of the experiment. None survived. The second fusion produced over 800 hybrid cell clusters in 182 wells, of which 21 wells were positive by day 9. The cells from 10 of these wells were recloned at 100 cells/well in 96 (small) well plates and the cells from about 15 positive (small) wells were pooled and frozen. The cells designated 1XA, 3B3, 6D6 and 7B1 were subsequently recloned further as spent medium from these cells demonstrated strong ELISA reactions on PDS-coated plates. A history of the manipulation of the cells from the 4 original wells to monoclonal status is presented in Table 5. Wells containing hybrid cell clusters or clones from plates of cells recloned at 10, 1 and 0.3 cells/well were invariably positive, with the amount of anti-PDS antibody present in the medium very often corresponding to the number and size of the clusters/clones present. When recloned at 1 cell/well, a positive well containing only a single, round hybrid cell cluster/clone was used as the source of cells to be recloned at 0.3 cells/well. At this latter dilution approximately 60 clones were expected to be found distributed amongst 192 small wells. In all of the situations in which the cells were recloned at 0.3 cells/well, less than one third of the expected number of clones was found and virtually all of these occurred singly in a well and were defined as clones. When clones were frozen and subsequently thawed, they were once again recloned at 0.3 cells/well.

The production of ascites fluid with each hybridoma was most successful in pristane-primed irradiated SJL/JxBalb/cCr male mice. Ascites fluid from hybridomas 3B3 and 6D6 could be obtained as many as 4-5 times from the same mouse whereas relatively little

TABLE 5
HISTORY OF HYBRID CELL LINES USED IN PROJECT

EVENT	1XA[1]	3B3[1]	6D6[1]	7B1[1]
Fusion	Feb/82	Feb/82	Feb/82	Feb/82
Recloned	100/well	100/well	100/well	100/well
Recloned		0.3/well		
Frozen	Mar/82	May/82	Mar/82	Mar/82
Thawed	June/82	Aug/82	July/82	June/82
Recloned	1/well	1/well	1/well	10/well
Recloned	0.3/well	0.3/well	0.3/well	1/well
Recloned				0.3/well
Frozen			Aug/82	Aug/82
Thawed			Oct/82	Oct/82
Recloned			1/well	1/well
Recloned			0.3/well	0.3/well
Ascites	Aug/82	Dec/82	Dec/82	Dec/82

[1]The history of each hybrid cell line in the table refers specifically to clones 1XA.4G.3F, 3B3.B.1B.4G.9E.3B.6D, 6D6.B.2D.1G.4E.2B and 7B1.3E.2G.2H.6D.3F which were used to produce ascites fluids. Supernatant fluids used in the experiments were derived from clones 3B3.B.1B.4G.9E.3B.6D, 6D6.B.2D.1G.4E.12B, 7B1.3E.2G.2H.6D.3E/3F (pooled), and 1XA.4G.7G.5G which was derived from 1XA.4G.7G, a thawed 0.3/well clone that had been recloned at 0.3 cells/well in Sept/82.

ascites fluid was obtained from mice bearing the hybridoma 7B1 before it became lethal.

3.3 Characterization of the Monoclonal Antibodies

3.3.1 Class, Subclass

Using class- and subclass-specific antisera in ELISAs, all 4 MAbs were found to be IgG_1 immunoglobulins. Both 6D6 and 7B1 were also eluted from protein A-Sepharose affinity columns with buffers characteristic of IgG_1 (Hudson and Hay, 1980), although it was discovered later by immunoblotting that the peroxidase conjugated rabbit anti-mouse IgG (H&L) antiserum appeared to recognize different determinants on these affinity purified MAbs (results not shown).

3.3.2 Competitive Binding Assays

These assays, designed to detect whether the 4 MAbs recognized different determinants on PDS, were carried out with different pair combinations of supernatant fluids from the 4 clones (Fig. 6). Only the combination of 1XA and 6D6 resulted in competitive binding (Fig. 6d). Use of ascites fluids in this assay provided the same results.

Clones 4C5.A.10B.9A.7A and 5D1.B.5G.7A.7H were also produced during the course of the project and their supernatant fluids were tested in competitive binding assays against the other 4 MAbs and each other. 5D1 was additive with all others, while 4C5 competed with both

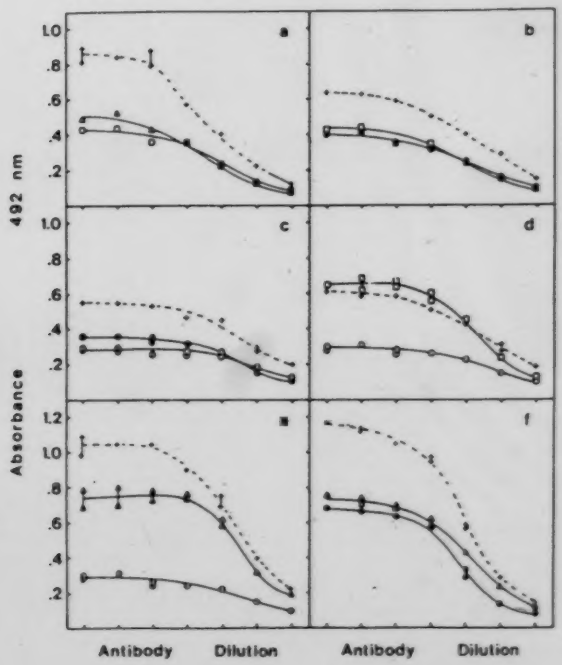

Fig. 6. Competitive binding ELISA assays. Hybridoma supernatant fluids were serially diluted (2-fold) individually, or pooled in pairs and diluted as mixtures, and incubated overnight at 6°C in PDS-coated wells to achieve saturation. After warming to room temperature, the ELISA was completed to determine whether mixtures of the MAbs from 2 different hybridomas could bind to the antigen in an additive fashion. 1XA (O), 3B3 (Δ), 6D6 (□), 7B1 (●) and the corresponding mixture of each pair (+). Panel a) 3B3 and 6D6, b) 6D6 and 7B1, c) 1XA and 7B1, d) 1XA and 6D6, e) 1XA and 3B3 and f) 3B3 and 7B1.

1XA and 6D6 (results not shown). As studies with the 4 other MAbs were under way, these antibodies were not used further.

3.3.3 Nature and Location of Epitopes on PDS

The nature and location of the epitopes on protodermatan sulfate were analyzed by ELISA inhibition assays and immunoblotting methods. The conditions of the ELISA inhibition assay were initially established using intact PDS as the standard inhibitor. It was found that 100,000 fold dilutions of 3B3, 6D6 and 7B1 ascites fluids gave sensitive, reproducible results as they were routinely inhibited to the 50% level by 20-60 ng PDS protein/ml depending upon the MAb used (see Figs. 7 & 8). 1XA ascites fluid, at the same dilution, required about 5-10 times more PDS protein/ml to be inhibited to the same degree and was therefore used at higher dilutions. Supernatant fluids of 6D6 and 7B1 cell cultures were also employed and, at dilutions of 1:2000 and 1:800, respectively, were inhibited by 10-30 ng PDS protein/ml (see Fig. 12).

All 4 MAbs also recognized intact PDS that had been denatured and reduced and subsequently electrophoresed from SDS-polyacrylamide slab gels to nitrocellulose paper (Plate 1). It was found that the use of 80 volts overnight (conditions used to blot collagen, for example) caused PDS to move through the nitrocellulose paper. Using 8 volts, the proteoglycan was adequately transferred (as judged by the lack of sample left in the gel) and typically appeared as a broad band on nitrocellulose paper stained by either Amido black or immunochemical means. Very often, PDS did not stain uniformly on immunoblots, but more intensely at the sides and weaker in the center of the lane. This may have been due to the electrophoresis conditions or, alternatively, due to an immunological prozone effect whereby the concentration of PDS or the primary antibody was too high in the central regions to permit maximal immunological reaction at the next step. The proteoglycan also displayed an average M_r that increased with an increase in the porosity of the gel: on 4% gels, it had an average M_r = 80,000-90,000 while on 7% gels it had an average M_r = 70,000. Its mobility remained the same whether the sample was heated to 45°C for 30 min or boiled for 5 min prior to

SDS-polyacrylamide gel electrophoresis. Likewise, its mobility did not change after heating in sample buffer containing 10% (v/v) 2-mercaptoethanol, 8M urea and 10% SDS. Using 6D6 in conjunction with serial dilutions of PDS in consecutive lanes, it was possible to detect as little as 30 ng of PDS protein per lane.

Both of these methods were subsequently used with all 4 MAbs to evaluate the antigenicity of PDS that had been treated enzymatically and/or chemically to remove the various carbohydrate side chains. Following chondroitinase ABC digestion, which removed the bulk of the dermatan sulfate side chain(s), all 4 MAbs recognized a major (Mr = 55,000) and a minor (Mr = 50,000) product, regarded as (ABC)cores, as well as a minor high molecular weight component having an Mr = 92,000-98,000 (Plate 2). Biotsed chondroitinase ABC did not stain with any of the MAbs (results not shown). In ELISA inhibition assays the purified (ABC)core preparation was found to be as effective an inhibitor for 3B3, 6D6 and 7B1 as the intact molecule (Fig. 7). For 1XA, the (ABC)core preparation was a somewhat less effective inhibitor and also produced an inhibition curve with a slope significantly different from that of intact PDS.

The monoclonal antibodies also recognized the protein core of PDS produced by a β-elimination, sulfite addition reaction which was supposed to have removed all O-linked carbohydrate side chains (Plate 3). Although some PDS appeared to have remained undigested, the major products of the reaction had electrophoretic mobilities very similar to those of the (ABC)cores of PDS. All 4 MAbs also recognized what appeared to be a single protein core of PDS after it had been treated with anhydrous HF (Plate 1), a procedure that removed virtually all of the carbohydrate side chains from the proteoglycan leaving only several N-acetylglucosamine residues N-linked to asparagine residues (Pearson et al., 1983). This protein core had an Mr = 43,000. All 4 antibodies recognized higher molecular weight components (Mr = 75,000 and Mr = 117,000 and others) and 1XA, 3B3 and 6D6 recognized a minor component (Mr = 26,000) as well (Plate 1). 6D6 also recognized the single (ABC)core of an antigenically related proteoglycan present in concentrated medium collected from confluent

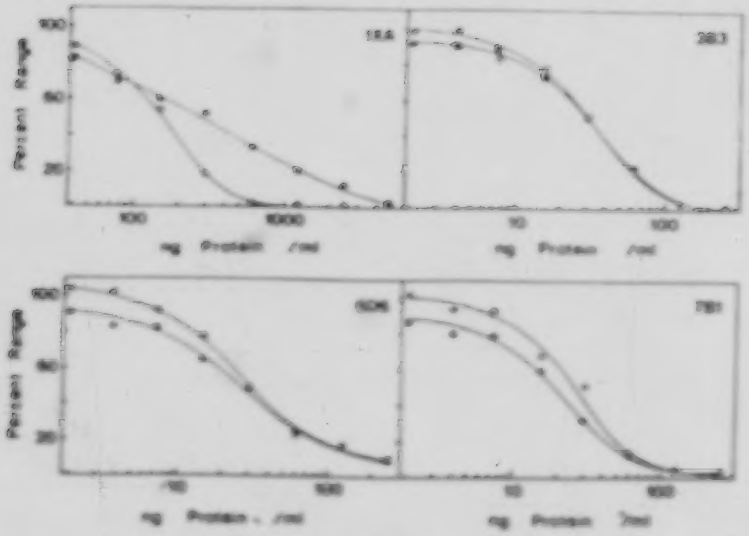

Fig. 7. ELISA inhibition assay with purified (ABC)core preparation of bovine skin PDS. The antigenicity of the (ABC)core preparation of PDS (●), produced by chondroitinase ABC digestion (0.2 units enzyme/mg sample at pH 7.4 in a stainless steel tube in the presence of proteinase inhibitors) and purified by chromatography on a Sephacryl S-300 column, was compared to undigested PDS (O) using ascites fluid derived from each of the 4 hybridomas.

human gingival fibroblasts that been incubated in the presence of tunicamycin (Plate 4); an agent that prevents attachment of N-linked oligosaccharides to the protein core during biosynthesis. It had an Mr = 44,000.

Proteodermatan sulfate was also treated with several enzymic and chemical proteolytic agents and the MAbs were used to evaluate the antigenicity of the resulting fragments. Cathepsin C digestion of PDS results in the sequential removal of dipeptides from the NH$_2$-terminus of the protein core, stopping after the first 6 amino acids, which includes removal of the dermatan sulfate side chain attached to residue 4 (Chopra and Pearson, unpublished results). Immunoblots of a cathepsin C digest of PDS (Plate 5a) indicated that 114, 383 and 6D6 recognized the resulting protein core that possessed an electrophoretic mobility equal to that of the (ABC)core(s) of PDS. Although poorly stained in this particular immunoblot, the ability of 7B1 to bind the cathepsin C-produced protein core was verified on

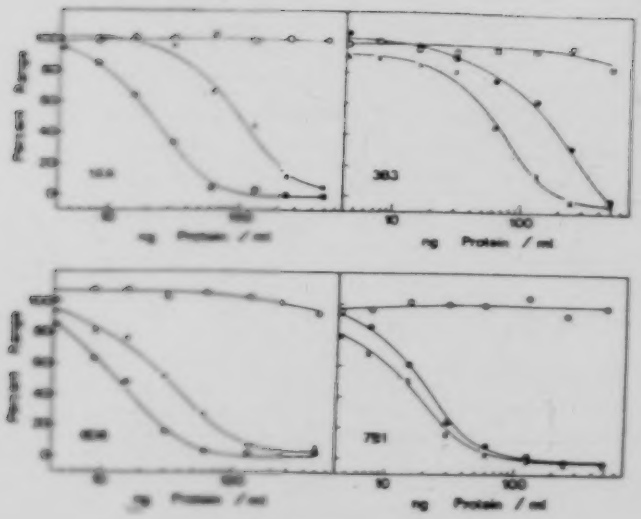

Fig. 8. ELISA inhibition assay with proteolytically degraded bovine skin PDS. Using hybridoma ascites fluids containing each monoclonal antibody, the antigenicity of PDS that had digested with papain for 24 hr at 65°C (O) was compared to that of PDS that had been incubated under the same conditions in buffer lacking papain (●) and to untreated PDS (X).

another immunoblot using aliquots from the same digest (Plate 5b).

Papain digestion of proteoglycans has long been used as a method to degrade the protein core as completely as possible in order to isolate the glycosaminoglycan side chains with relatively little protein attached. Using ELISA inhibition assays, it was demonstrated that papain digestion of PDS resulted in a marked loss of antigenicity for all 4 MAbs (Fig. 8). However, it was also found that PDS became a more effective inhibitor for 1XA and 6D6 (than untreated PDS) after it had been incubated for 24 hr at 65°C in buffer lacking papain (*i.e.*, in the control digest, Fig. 8). Conversely, the PDS in the control digest proved to be a less effective inhibitor for 3B3 than untreated DS. By eliminating components from the buffer, it was found that the presence of cysteine HCl (an activator for papain) caused this effect; prolonged incubation at 65°C did not alter the antigenicity of PDS for 3B3 (results not shown). 3B3 was inhibited by the control digest untreated PDS to almost the same extent (Fig. 8).

PDS that had been digested with trypsin also resulted in a loss of antigenicity for 1XA, 6D6 and 7B1 as revealed by both immunoblotting (Plate 6) and ELISA inhibition assays (results not shown). 3B3, however, recognized a small peptide produced by trypsin on the the immunoblot (lane 7, Plate 6) and was the only MAb to be inhibited (slightly) by the trypsin digest of PDS.

PDS that had been digested with cathepsin D was also analyzed by immunoblotting. Cathepsin D causes only limited proteolysis of PDS (Pearson, unpublished results) and all 4 MAbs recognized various enzyme-produced fragments (Plates 6 & 7). Antibody 7B1 recognized a broad band having an average Mr = 45,000 (Plate 6) which, after chondroitinase ABC digestion, resulted in the MAb recognizing 2 peptides with Mr = 17,500 and Mr = 24,000 (Plate 7). Antibodies 1XA and 6D6 also recognized two fragments produced by cathepsin D (Mr = 25,000 and Mr = 15,000), the smaller being a major product as detected by Amido black. It also stained faintly with Periodic-acid Schiff (Plate 7).

An immunoblot of the CNBr peptides of PDS (Plate 8) revealed that the peptides recognized by 1XA and 6D6 corresponded to the two major cyanogen bromide peptides (Mr = 35,000 and Mr = 30,000) stained by Amido black. These peptides were also stained by PAS (Plate 7). Antibody 3B3 did not appear to recognize the major Amido black-staining peptides, but others apparently present in low amounts. Antibody 7B1 did not bind to any of the cyanogen bromide peptides in this immunoblot, but when the peptides were fractionated with CPC, it did recognize a CPC-precipitable component that ran as a broad band having an average Mr of approximately 45,000 (results not shown). This was also apparent in Plate 7. When the cyanogen bromide peptides were treated with chondroitinase ABC, 7B1 recognized a peptide with Mr = 19,000 (Plate 7). Similarly, an immunoblot of the cyanogen bromide peptides of the (HF)-core of PDS resulted in a peptide with Mr = 19,000 (Plate 9) recognized by 7B1.

The results described above suggested that the epitope of 7B1 was located on a dermatan sulfate-containing cyanogen bromide peptide. To confirm this, the dermatan sulfate-containing cyanogen bromide peptide(s) were isolated, first by ion exchange chromatography in

Fig. 9. Bovine skin PDS (18.95 mg, preparation 10), previously reduced, was digested with cyanogen bromide under N_2 for 4 hr at 30°C. 3.86 mg of the digest was dissolved in 220 µl of a 7M urea, 0.15M NaCl, 0.05M Tris HCl, pH 6.5 buffer and injected onto a 1 x 10 cm Polyanion SI-17 column coupled to an FPLC system. The column was eluted with the same buffer until the absorbance at 230 nm returned to baseline at which time elution with a programmed linear gradient of NaCl commenced using a progressively increasing amount of buffer B (7M urea, 2.0M NaCl, 0.05M Tris HCl, pH 6.5). 80 µl of fractions 55-90 were diluted 3.75-fold and analyzed for uronic acid content on a Technicon Autoanalyzer. 75% of the uronic acid injected onto the column was recovered. Fractions containing uronic acid were pooled as indicated and precipitated with 98% ethanol. The arrow indicates the elution position of intact PDS.

Fig. 10. The ethanol precipitate of the pooled uronic acid-containing fractions previously eluted from the Polyanion SI-17 column were dissolved in 1 ml of a 7M urea, 0.3M NaCl, 0.05M Tris HCl, pH 7.2 buffer and applied to a calibrated 1 x 111 cm Sephacryl S-300 column and eluted with the same buffer at 6 ml/hr. Fractions 32-60 and 88-92 were analyzed for uronic acid content and fractions 47-60 were pooled as indicated, dialyzed against water and lyophilized.

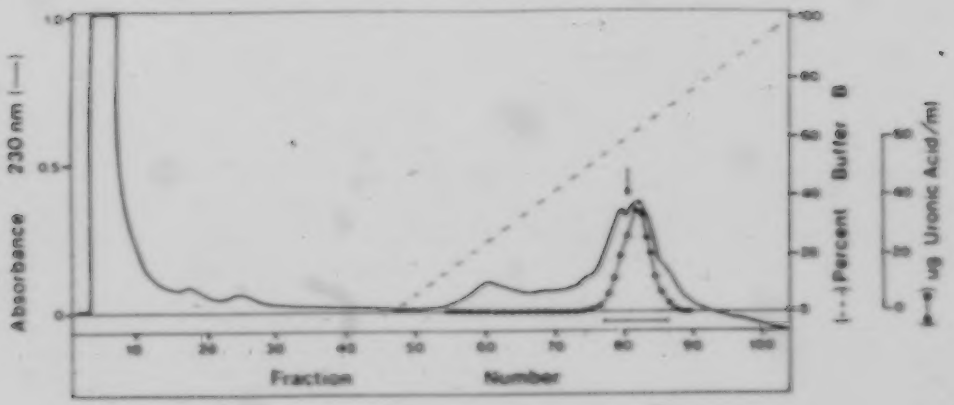

Figure 9.

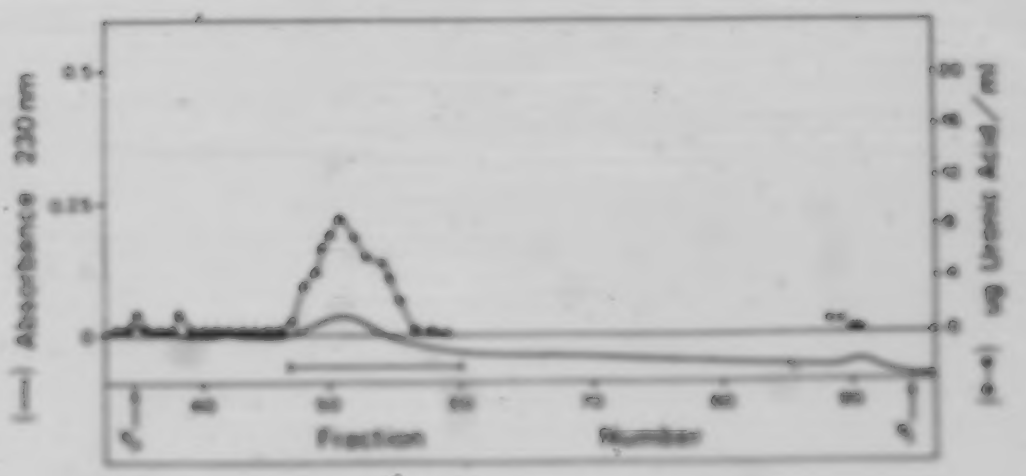

Figure 10.

TABLE 6
AMINO ACID ANALYSIS OF DERMATAN SULFATE-CONTAINING CYANOGEN BROMIDE PEPTIDE OF PROTEODERMATAN SULFATE

Amino Acid	Isolated CB Peptide[1] (Residues/21)	Expected CB Peptide[2] (Residues/21)
Asx	1.3	1
Thr	0.2	
Ser	1.3	1
HSer	0.7	1
Glx	6.0	6
Pro	3.9	4
Gly	2.1	2
Ala	1	1
Cys	-	
Val	0.9	1
Met	-	
Ileu	1.5	2
Leu	0.2	
Tyr	0.1	
Phe	0.9	1
Lys	0.2	
His	0.8	1
Arg	0.1	

[1]Total Acid Hydrolysis, average of 2 analyses
[2]From known amino acid sequence described by Pearson et al., 1983.

conjunction with a linear salt salt gradient (Fig. 9) and then gel filtration chromatography (Fig. 10). Both chromatographic methods used buffers containing 7M urea. The purity of the glycosaminoglycan-bearing peptide(s) was assessed by comparing the amino acid composition of the preparation with the known amino acid content of the NH_2-terminal cyanogen bromide peptide (CB-1) bearing the glycosaminoglycan (Table 6). Based on the content of homoserine, approximately 70% of the preparation was CB-1. When the preparation was electrophoresed in nitrocellulose paper at pH 8.6, 7B1 recognized a glycosaminoglycan-containing peptide, that migrated with virtually the same mobility as the dermatan sulfate standard (Plate 10). When the insoluble fraction of a cyanogen bromide digest was electrophoresed in nitrocellulose paper, 7B1 was the only MAb to stain the single component (as detected by Toluidine blue staining) that

3.4 Specificity of the Monoclonal Antibodies

3.4.1 Crossreactivity with Other Connective Tissue Components of Skin

Using ELISA inhibition analyses, none of the MAbs was inhibited by bovine fibronectin (Fig. 11), 1000 ng of bovine elastin or 5000 ng/ml of commercially available hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate or dermatan sulfate. Bovine types I, III and IV collagen did not inhibit the MAbs (Fig. 12). Bovine type II collagen was also tested and gave similar results (Fig. 12). None of the MAb supernatant fluids bound bovine acid soluble type I collagen coated ELISA plates, but when 50 μg of bovine fetal calf skin type I collagen was subjected to immunoblotting with 6D6, very small amounts of contaminating PDS were detectable (results not shown).

3.4.2 Crossreactivity with Other Purified PDS Preparations

L-iduronic acid-rich proteodermatan sulfate had previously been isolated from bovine periodontal ligament and partially characterized (Pearson & Gibson, 1982). On immunoblots all

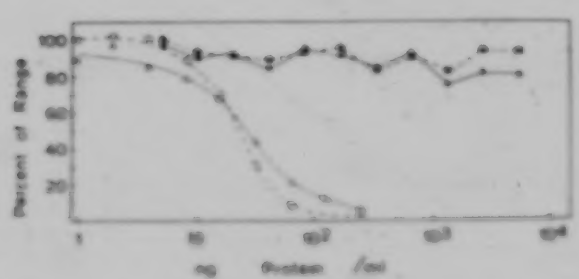

Fig. 11. ELISA inhibition assay with bovine fibronectin, 6D6 and 7B1 ascites fluids, diluted 10^3-fold, were incubated overnight at 6°C with intact bovine skin PDS (▲ and □ , respectively) or bovine fibronectin (▲ and ■ , respectively) and transferred to PDS coated wells. The points represent the proportion of uninhibited MAb at each inhibitor concentration. 1XA and 3B3 ascites fluids were not inhibited by 5000 ng/ml of fibronectin.

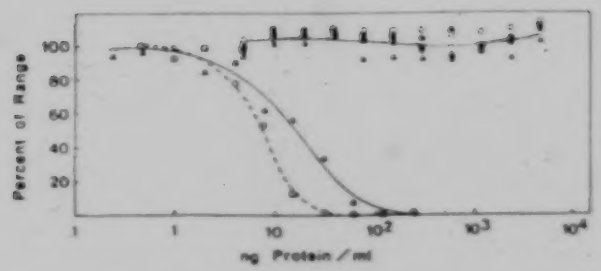

Fig. 12. ELISA inhibition assay with bovine collagens. 6D6 supernatant fluid (1:2800) and 7B1 supernatant fluid (1:800) were incubated overnight at 6°C with PDS (△ and □ , respectively) or bovine types I (○), II (●), III (■) or IV (▲) collagen and then transferred to PDS-coated wells. 1XA and 3B3 monoclonal antibodies were also uninhibited by 5000 ng/ml of the collagens.

4 MAbs recognized periodontal ligament PDS and its (ABC)core preparation (Plate 12). The major (ABC)core produced was the same size as that of skin PDS. However, the intact proteoglycan appeared to be larger, having an Mr = 110,000. Immunoblots of bovine gingiva PG2, the uronic acid-containing component that eluted as peak 3 from the Sepharose CL-4B column (Plate 13), and bovine embryonic skin PDS (Plate 14) provided the same results. The pattern of staining obtained with human gingiva PDS resembled bovine gingiva PDS (Plate 15), but was bound negligibly by 7B1 (significantly, this immunoblot used 1% bovine serum albumin rather than 2% and was the only occasion that normal mouse serum appeared to bind to a component). Adult human skin PDS, extracted using hot 6M urea, was recognized by 1XA, 3B3 and 6D6 only (Plate 16) and was the only intact PDS studied which had a mobility similar to adult bovine skin PDS (Plate 16). Its protein core, when prepared by treatment with alkali, was not recognized by the MAbs.

3.4.3 Cross-reactivity with Proteoglycans from Other Sources

As described above, 6D6 recognized the (ABC)core of a proteoglycan present in concentrated culture medium of confluent human gingival fibroblasts that had been treated with

3.4.3 Crossreactivity with Proteoglycans from Other Sources

As described above, 6D6 recognized the (ABC)core of a proteoglycan present in concentrated culture medium of confluent human gingival fibroblasts that had been treated with tunicamycin (Plate 4). An immunoblot of the concentrated medium obtained from untreated human gingival fibroblasts revealed that 1XA, 3B3 and 6D6 recognized the intact proteoglycan (Mr = 110,000) and its two distinct (ABC)cores (Plate 17), which displayed the same mobility as the (ABC)cores derived from bovine skin PDS. However, the two gingival fibroblast (ABC)cores were present in more equal poportions. 7B1 did not recognize either component. Concentrated medium collected from cultures of confluent human embryonic skin fibroblasts and human adult skin fibroblasts also contained an antigenically related proteoglycan (Mr = 110,000) with the same (ABC)core(s), as detected by 6D6 (results not shown). Antibodies 3B3 and 6D6 (and possibly 1XA as well) also recognized a proteoglycan (Mr = 135,000) and its (ABC)core (Mr =43,000) in concentrated culture medium collected from monkey arterial smooth muscle cells (Plate 18).

To determine whether any of the MAbs crossreacted with the large cartilage-specific proteoglycan monomer, A1D1 fractions extracted from rat chondrosarcoma and bovine nasal cartilage were analyzed by ELISA inhibition and immunblotting methods. This fraction was produced by the method of Sajdera and Hascall (1969). The high buoyant density components of a cartilage extract produced by centrifugation in a CsCl density gradient under associative conditions (i.e., 0.4M GdnHCl), and dsignated fraction A1, were then recentrifuged in a CsCl density gradient under dissociative conditions (i.e., 4M GdnHCl). Fraction A1D1 represents the high buoyant density components from action A1 and has been shown to contain the cartilage-specific monomer. In ELISA inibition assays, only 1XA was slightly inhibited by rat chondrosarcoma A1D1 and its (ABC)core preparation (Fig. 13), although none of the MAbs recognized 14 μg (protein) of rat A1D1 or its (ABC)core on immunoblots (Plate 19). Failure of 1XA to recognize the major A1D1 components could not have been due to a lack of material binding actess to the nitrocellulose gags. In fact, so much of the (ABC)core of the

Fig. 13. ELISA inhibition assay with the A1D1 fraction from the Swarm rat chondrosarcoma. Using ascites fluids of 1XA (1:400,000) and 3B3, 6D6 and 7B1 (1:200,000), the antigenicity of the A1D1 fraction (7% protein) (○) and an A1D1 preparation that had been incubated with 0.2 units of chondroitinase ABC/mg sample for 8 hr at 37°C (■), was compared to that of bovine skin PDS (●). The samples were boiled for 5 min prior to their serial dilution in PBS/T. The MAb-inhibitor solutions were incubated at 6°C for 40 hr prior to being transferred to PDS-coated wells and completing the assay. The points plotted represent the average of duplicate analyses.

Fig. 14. ELISA inhibition assay with the A1D1 fraction extracted from bovine nasal cartilage. Using ascites fluids of 1XA (1:400,000) and 3B3, 6D6 and 7B1 (1:200,000), the antigenicity of the A1D1 fraction (6% protein) (○) and an A1D1 preparation previously incubated with chondroitinase ABC (0.2 units/mg sample) for 8 hr at 37°C (■), was compared to that of bovine skin PDS (●). The samples were boiled for 5 min prior to serially diluting them in PBS/T. The MAb-inhibitor solutions were incubated for 40 hr at 6°C prior to being transferred to PDS-coated wells and completing the assay. The points represent the average of duplicate analyses.

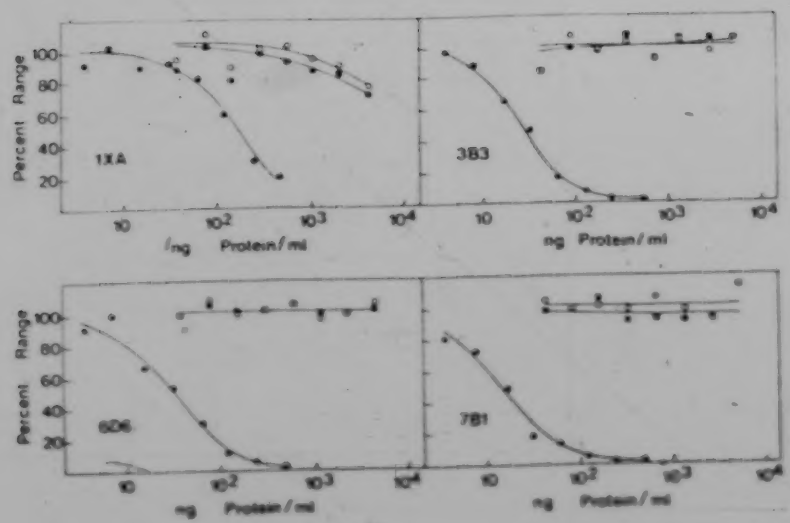

Figure 13.

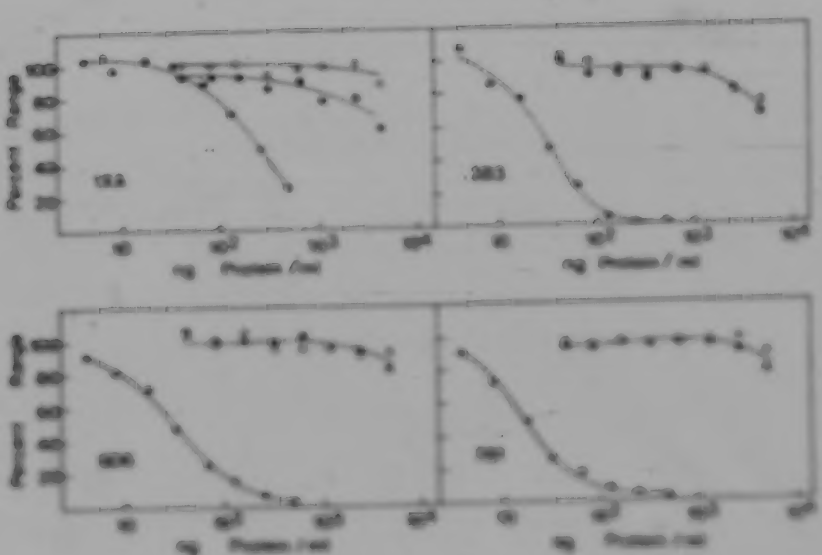

Figure 14.

Fig. 15. ELISA inhibition assay with the A1D4 fraction isolated from Swarm rat chondrosarcoma. Using ascites fluids of 1XA (1:200,000) and 3B3, 6D6 and 7B1 (1:100,000), the antigenicity of the A1D4 fraction (26% protein) (○) and an A1D4 preparation that had been incubated with chondroitinase ABC (0.2 units/mg sample) for 8 hr at 37C (■), was compared with bovine skin PDS (●). The samples were boiled for 5 min prior to serially diluting them in PBS/T. The MAb-inhibitor solutions were incubated for 40 hr at 6℃ prior to transferring them to PDS-coated wells. The points represent the average of duplicate analyses.

Fig. 16. ELISA inhibition assay of the A1D4 fraction extracted from bovine nasal cartilage. Using ascites fluids 1XA (1:400,000) and 3B3, 6D6 and 7B1 (1:100,000), the antigenicity of the A1D4 fraction (48% protein) (○) and an A1D4 preparation that had been incubated with chondroitinase ABC (as described above) (■), was compared to that of bovine skin PDS (●). The samples were boiled for 5 min prior to serially diluting them in PBS/T. The MAb-inhibitor solutions were incubated for 40 hr at 6℃ prior to transferring them to PDS-coated wells. The plotted points represent the average of duplicate analyses.

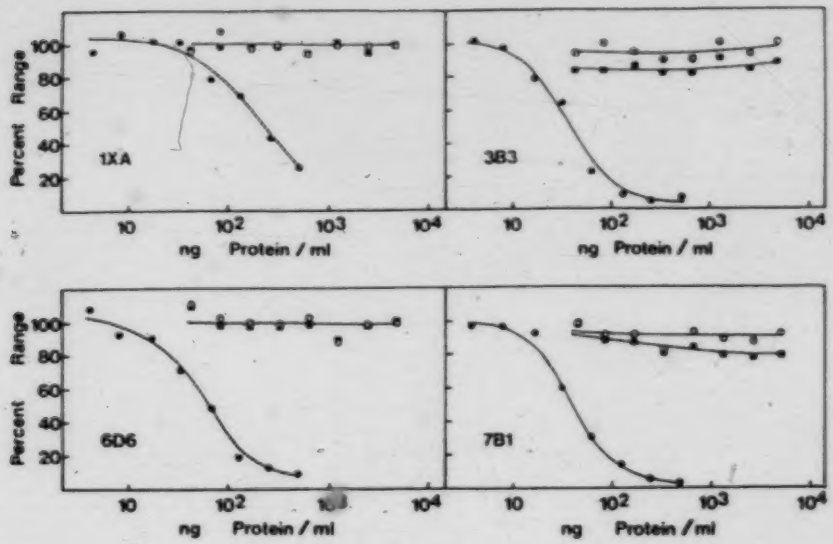

Figure 15.

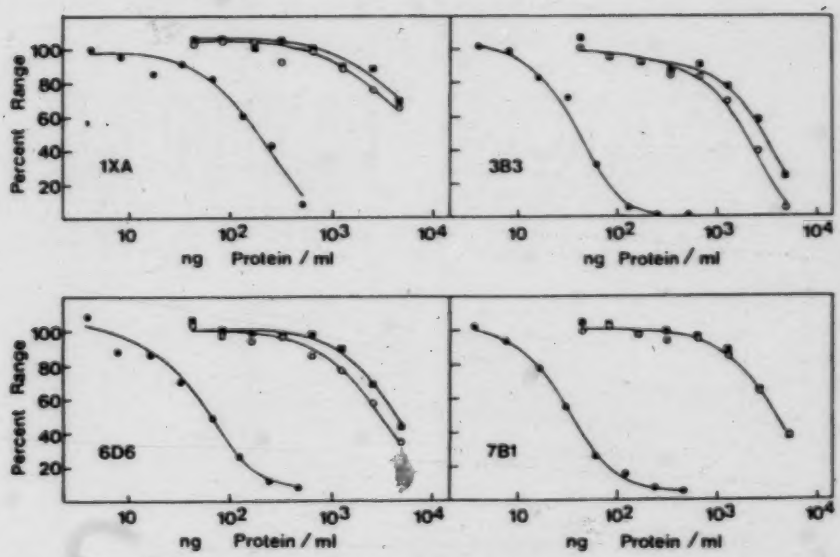

Figure 16.

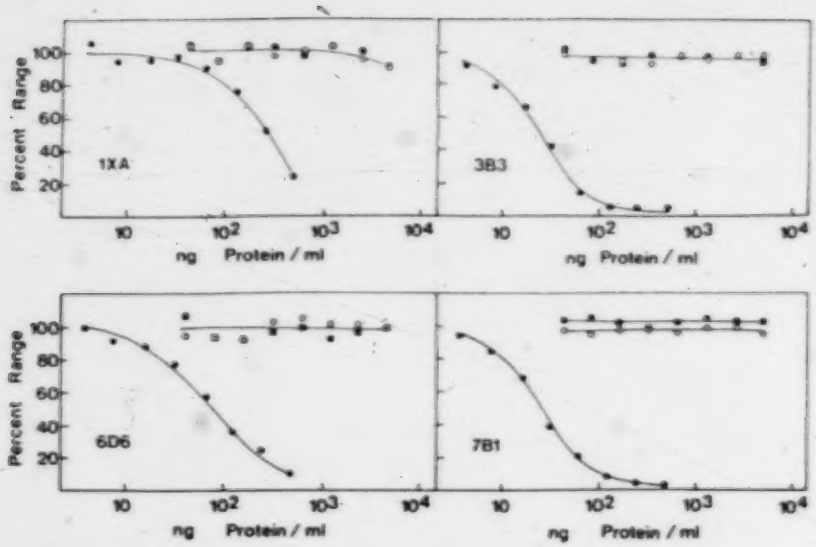

Fig. 17. ELISA inhibition assay with the A1D4 fraction extracted from chick sternal cartilage. Using ascites fluids 1XA (1:400,000) and 3B3, 6D6 and 7B1 (1:200,000), the antigenicity of the A1D4 fraction (50% protein) (○) and an A1D4 preparation that had been incubated with chondroitinase ABC (as described previously) (■), was compared to that of bovine skin PDS (●). The samples were boiled for 5 min prior to dilution with PBS/T. The MAb-inhibitor solutions were incubated for 40 hr at 6°C prior to transferring them to warmed PDS-coated wells.

of the preparation revealed that the MAbs did not bind to the large proteoglycan or its (ABC)core, but to a much smaller proteoglycan similar to those in bovine periodontal ligament and bovine gingiva (Plate 20).

To determine whether any of the MAbs crossreacted with the link proteins extracted from the various cartilages, the low buoyant density components of fraction A1 (i.e., fraction A1D4) were also analyzed with the MAbs. None recognized rat chondrosarcoma A1D4, either before or after chondroitinase ABC digestion, in ELISA inhibition assays (Fig. 15) or on immunoblots (Plate 21). However, all 4 antibodies were inhibited to a significant degree by bovine nasal cartilage A1D4, both before and after chondroitinase ABC digestion (Fig. 16). An immunoblot of unreduced bovine A1D4 revealed that all 4 MAbs recognized a proteoglycan,

immunoblots (Plate 21). However, all 4 antibodies were inhibited to a significant degree by bovine nasal cartilage A1D4, both before and after chondroitinase ABC digestion (Fig. 16). An immunoblot of unreduced bovine A1D4 revealed that all 4 MAbs recognized a proteoglycan, apparently present in substantial amounts, with a mobility (Mr=105,000) similar to that of the small antigenically-related proteoglycan in bovine cartilage A1D1 (Plate 22). Chicken sternal cartilage A1D4 appeared to inhibit only 1XA (Fig. 17) which was also the only MAb to recognize an (ABC)core in that preparation (Plate 23). None of the MAbs recognized the major Amido black-staining components of either A1D4 preparation, the link proteins.

Bovine dental pulp PG2, a uronic acid-containing component that eluted from the Sepharose CL-4B column in the same position as that of the other purified proteodermatan sulfates (ie., peak 3) was also analyzed by ELISA and immunoblotting methods. All 4 MAbs were inhibited to some extent by the bovine pulp PG2 preparation (Fig. 18) and on immunoblots all 4 antibodies recognized a proteoglycan (Mr=115,000) possessing (ABC)core(s) similar in mobility to those of bovine skin PDS (Plate 24). However, none of the antibodies recognized the major component of the preparation (as detected by Amido black staining); a proteoglycan (Mr=135,000) that possessed a high molecular weight (ABC)core (Mr=100,000). While all the MAbs were inhibited by the larger bovine dental pulp PG1 preparation (Fig. 19), immunoblots revealed that they did not bind to the large proteoglycan or its (ABC)core (Mr>200,000), but did recognize a proteoglycan that was similar in size to the smaller antigenically-related proteoglycan present in pulp PG2 (Plate 25). Numerous higher molecular weight components antigenically related to PDS were also evident in pulp PG1.

3.5 Nature of Antigenically Related High Molecular Weight Components in PDS Preparations

In a number of the immunoblots presented thus far, the MAbs recognized components that possessed apparent molecular weights greater than intact PDS or its (ABC)core. Of particular concern was a 92,000-98,000 Mr component present in immunoblots of the (ABC)cores of PDS and antigenically related moieties. Although present in low levels (i.e., less

Fig. 18. ELISA inhibition assay with bovine dental pulp PG2 (preparation 5D). Using hybridoma ascites fluids 1XA (1:400,000) and 3B3, 6D6 and 7B1 (1:100,000), the antigenicity of bovine dental pulp PG2 (O) and PG2 incubated with chondroitinase ABC (0.1 unit/mg sample) (■), was compared to that of untreated bovine skin PDS (●). The samples were boiled for 5 min prior to dilution in PBS/T. The plotted points represent the average of duplicate analyses.

Fig. 19. ELISA inhibition assay with bovine dental pulp PG1 (preparation 7A). Using hybridoma ascites fluids of 1XA (1:400,000) and 3B3, 6D6 and 7B1 (1:200,000), the antigenicity of bovine pulp PG1 (O) and pulp PG1 that had been incubated with chondroitinase ABC (0.1 units/mg sample) (■), was compared with to that of untreated bovine skin PDS (●). The samples were boiled for 5 min prior to dilution in PBS/T. The points represent the average of duplicate analyses.

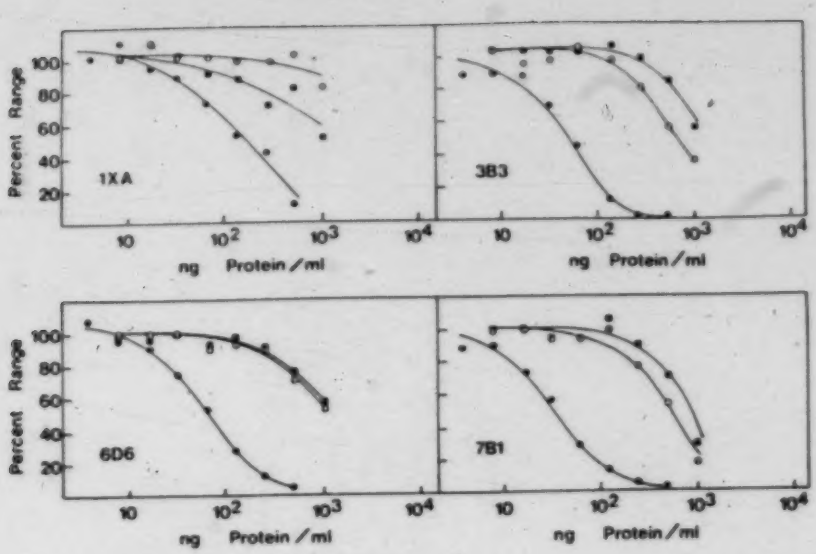

Figure 18.

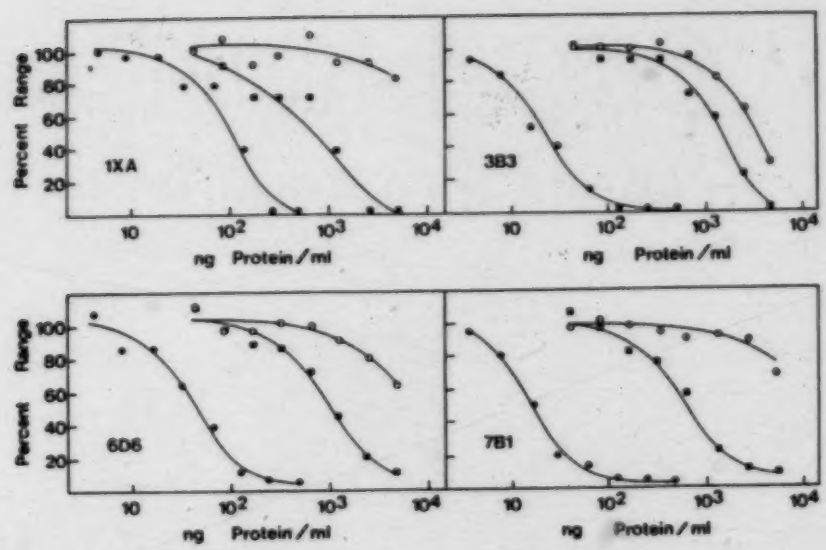

Figure 19.

than 5-10%), its apparent resistence to reduction, the uncertainty as to whether it was masked in undigested preparations by PDS itself and its apparent molecular weight raised questions as to its structure and whether it was derived from PDS. To get some understanding of this component, a dialyzed and lyophilized pooled sample of PDS, that had been chromatographed on a calibrated 1 x 112 cm Sephacryl S-300 column (using a 4M GdnHCl, 0.5M sodium acetate, pH 6.8 buffer) to remove any significantly higher molecular weight components (results not shown), was digested with 0.25 units of chondroitinase ABC/mg sample and, after adding solid GdnHCl to render the solution 4M, was rechromatographed on the same 1 x 112 cm Sephacryl S-300 column (Fig. 20). Three distinct peaks, detected by absorbance at 280 nm, were observed. An immunoblot of ethanol precipitated fractions 34, 41 and 48 (with and without prior reduction) suggested that peak 1 and peak 2 contained multimers of the (ABC)core stabilized by intermolecular disulfide bonds (Plate 26). Significantly, fraction 41 (i.e., peak 2) retained the 92,000-98,000 Mr component after reduction. In an immunoblot of another (ABC)core preparation, the amount of this component was apparently decreased by heating in sample buffer containing 8M urea/10% SDS (Plate 27).

3.6 Immunohistochemical Localization of PDS

3.6.1 Light Microscopic Studies

At the light microscopic level PDS was immunohistochemically localized employing the peroxidase anti-peroxidase method. Using formalin fixed, dewaxed sections of mature bovine skin in conjunction with 6D6 supernatant fluid the oxidized diaminobenzidine precipitate was found to be present throughout the connective tissue regions of skin but absent from the epithelial structures such as hair follicles, sweat and sebaceous glands and their ducts (Plate 28a). The stain was most prominent in the papillary (superficial) layer of the dermis and between and around the large collagen fiber bundles of the reticular (deep) layer. Where epidermis had not been completely scraped away, none of the basal epidermal cells were stained,

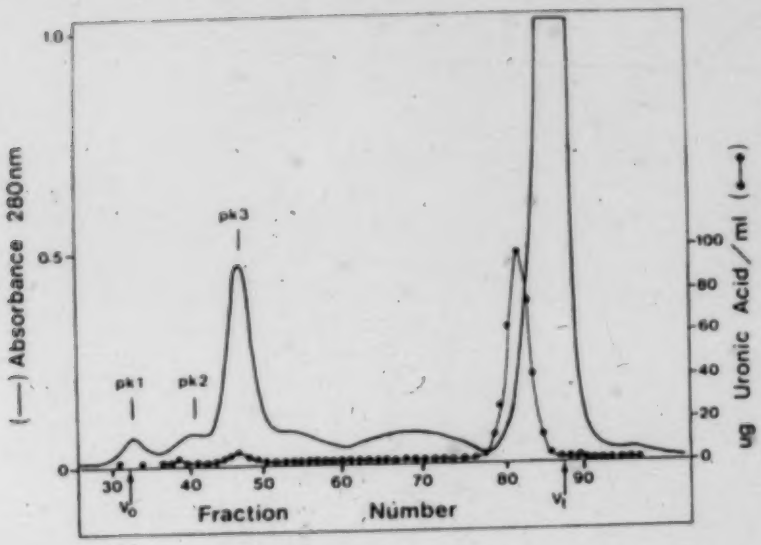

Fig. 20. Sephacryl S-300 column chromatography of a bovine skin PDS (ABC)core preparation. 4.5 mg of bovine skin PDS (previously chromatographed on the same Sephacryl S-300 column under dissociative conditions, using a 4M guanidinium chloride, 0.5M sodium acetate, pH 6.8 buffer) was dissolved in 1 ml of 0.1M sodium acetate, 0.1M TSis HCl, pH 7.4 buffer and digested with 1.0 unit of chondroitinase ABC for 45 min at 37C. 382 mg of guanidinium chloride was added to the digest, and once dissolved, the digest was microfuged for 5 seconds and the supernatant applied to the calibrated 1 x 112 cm Sephacryl S-300 column. The sample was eluted with 4M guanidinium chloride, 0.5M sodium acetate, pH 6.8 buffer at ~6 ml/hr. 80 μl aliquots of fractions were diluted and analyzed for uronic acid content.

component, a dialyzed and lyophilized pooled sample of PDS, that had been chromatographed on a calibrated 1 x 112 cm Sephacryl S-300 column (using a 4M GdnHCl, 0.5M sodium acetate, pH 6.8 buffer) to remove any significantly higher molecular weight components (results not shown), was digested with 0.25 units of chondroitinase ABC/mg sample and, after adding solid GdnHCl to render the solution 4M, was rechromatographed on the same 1 x 112 cm Sephacryl S-300 column (Fig. 20). Three distinct peaks, detected by absorbance at 280 nm, were observed. An immunoblot of ethanol precipitated fractions 34, 41 and 48 (with and without prior reduction) suggested that peak 1 and peak 2 contained multimers of the (ABC)core stabilized by

between the fibers in bundles cut approximately in cross-section. In relation to the finer fibers of the connective tissue matrix, the stain was typically more intense. When sections were counterstained with Alcian blue, a few fibroblasts appeared to stain for PDS (results not shown). In control sections using normal mouse serum faint staining was confined to the epithelial structures of skin (Plate 28d). With another control, supernatant fluid derived from the myeloma parent cell line, staining was completely absent.

Use of 3B3 and 7B1 supernatant fluids resulted in a pattern of staining indistinguishable from that described above. Neither 1XA supernatant fluid or ascites fluid at various dilutions stained fixed tissue sections well. However, all four antibodies effectively stained frozen, unfixed sections of bovine skin and in a similar manner to that described above for the fixed sections (results not shown).

PDS was also located immunohistochemically in fixed sections of fetal calf skin (Plate 29a). Using such criteria as dermal and epidermal thickness and hair follicle development in conjunction with knowing the region from which the skin sample had been taken (Lyne and Heideman, 1959), the calf was estimated to be 130 days of age. Like the adult skin sections, this tissue stained well by 3B3, 6D6 and 7B1 only, but unlike the adult skin sections the stain was largely confined to the deeper layers of the dermis with very little in the subepidermal zone (Plate 29a). Treatment of the tissue sections with hyaluronidase did not enhance the staining within this region (results not shown). When the enzyme reaction was allowed to continue for an extended period of time stain was eventually deposited in the superficial layer, but only faintly and in a fine reticular pattern in the connective tissue between the epithelial structures (Plate 29b). As in the adult tissue, only the epithelial structures were stained in the controls (Plate 29c).

Other fetal calf skin samples whose original position was unknown were obtained and stained with 6D6. When the samples were arranged in an age sequence based on the degree of development of the hair follicles (Lyne & Heideman, 1959), it was apparent that the stain became more intense in the papillary layer with increasing age. It also followed a distinct pattern

TABLE 7
CROSSREACTIVITY OF MONOCLONAL ANTIBODIES WITH PDS IN SKIN FROM VARIOUS SPECIES

Tissue	Control	1XA	3B3	6D6	7B1
adult bovine skin, frozen	-	++	++	++	++
adult bovine skin, fixed	-	±	++	++	+
fetal calf skin, frozen	-	++	++	++	++
fetal calf skin, fixed	-	±	++	++	++
human gingiva, frozen	-	++	++	++	-
human skin, fixed	-	±	+	++	-
porcine skin, frozen	-	++	++	++	+
porcine skin, fixed	-	-	+	++	-
chicken skin, frozen	-	++	+	++	-
chicken skin, fixed	-	-	+	+	-
rabbit skin, fixed	±	+	++	++	±
monkey skin, fixed	±	±	++	++	-

Two fold dilutions of supernatant fluid from hybridomas 1XA, 3B3, 6D6 and 7B1 grown in culture were tested for their ability specifically to locate PDS in skin sections from several different species. The location of stain in adult bovine skin was used as the reference for specific staining (Plate 28a). The pattern of staining seen in Plate 28d represented nonspecific staining. Normal mouse serum diluted 100 fold was used as a control. Explanation of Symbols: (-), very faint nonspecific staining or no staining at all; (±), weak nonspecific and specific staining present; (+), weak to moderate specific staining; (++), moderate to strong specific staining.

(starting with Plate 29a) in which it increased first immediately below the epidermis (Plate 30a) and then gradually spread to the deeper layers surrounding the hair follicles themselves (Plate 30b-d).

The dermis of mature human skin was also examined immunochemically and, with 6D6 and 3B3, closely resembled bovine skin (Plate 31). Like formalin fixed bovine skin sections 1XA did not stain fixed human skin well, but 7B1 did not stain human skin sections at all (results not shown). The capability of each MAb to stain sections of skin from several other species was also

investigated and is summarized in Table 7.

Gingiva and tendon are also very fibrous tissues, rich in type I collagen. Immunohistochemical treatment of fixed sections of bovine gingiva with the MAbs (as seen with 3B3; Plate 32) resulted in a pattern of staining very similar to that found in sections of fixed bovine skin with stain deposited on and between the numerous collagen fiber bundles of the connective tissue regions but lacking in the epithelial structures, which stained only with normal mouse serum (results not shown). Frozen, unfixed sections of human gingiva were heavily stained following incubation with 1XA (Plate 33a), 3B3 and 6D6, but were not stained with 7B1 (Plate 33b). Sections of formalin fixed bovine tail tendon were also stained by the MAbs in characteristic fashion and, as with the thicker collagen fiber bundles in bovine skin, the large fiber bundles in tendon sections cut longitudinally did not stain intensely (Plate 34). The connective tissue layer investing the collagen fiber bundles of tendon were more intensely stained.

Dental pulp, consisting of a very gel-like connective tissue matrix, was also examined. When fixed sections of dental pulp from an unerupted bovine incisor were treated immunohistochemically with 6D6, the fine fibrous elements of the matrix were stained, although the stain was confined to the incisal 2/3-3/4 of the tissue (Plate 35).

Fixed sections of bovine nasal cartilage were also treated immunohistochemically with the MAbs. Incubation of the tissue sections with the MAbs resulted in characteristic staining of the connective tissue component of the superficial nasal tissue and the perichondrium; the hyaline matrix of the cartilage did not stain (Plates 36a and 36b). As with the sections of fetal calf skin, pretreatment of these sections with testicular hyaluronidase did not increase the amount of stain within the cartilage matrix. On the other hand, it caused a noticeable decrease in the staining of the nasal tissue with 3B3 and a loss of staining with 1XA (results not shown).

3.6.2 Electron Microscopic Studies

Monoclonal antibodies 6D6 and 7B1 were chosen for use in these studies as it had become apparent (see discussion) that they had different specificities and recognized epitopes in different regions of the protein core of PDS. Bovine skin samples were initially used in these studies but due to the irregularly arranged collagen fibers in dermis, it proved to be difficult consistently to obtain sections with collagen fibrils cut longitudinally. As a result, mature bovine tail tendon, with its more regularly arranged collagen fibers, was employed.

Using 6D6 or 7B1 with the peroxidase anti-peroxidase method, when the enzyme reaction was limited to 5 minutes, the oxidized diaminobenzidine precipitated as small, rather discrete particles confined to the surface of the collagen fibrils (Plate 37a-d). On fibrils cut longitudinally, the majority of the particles appeared to be associated with the collagen in a periodic fashion, located at and/or just to one side (usually the same side) of the single major collagen band per D period. Fibrils of all sizes observed were outlined by the small particles. Use of normal mouse serum (Plate 38a), 30% NGS alone (Plate 38b) or MAbs that had been preadsorbed with PDS (Plate 38c) resulted in a greatly decreased precipitate.

When tendon samples were treated with the MAbs in conjunction with the immunoferritin method, the presence of electron dense particles along the surface of the fibrils was much less frequent. However, their much smaller size and consistent shape made it possible accurately to locate the ferritin particles with respect to the collagen bands after staining with uranyl acetate (Plates 39 & 40). All nonspecific binding of ferritin to the collagen was effectively eliminated by repeatedly washing the samples in TBS following incubation with the ferritin conjugated antiserum, as shown in the control using normal mouse serum (Plate 39c). The names and positions of the bands, and their corresponding interband spaces, to which single ferritin molecules were assigned are shown in Fig. 21. Aggregates and small groups of ferritin molecules were not counted. The distribution of ferritin particles in each location is presented in Table 8 and as a histogram in Fig. 22. The frequency of ferritin particles located within zones *d* and *e* differed from the predicted mean by 1.3 S.D. while the frequency in zone *b* differed by 1.5

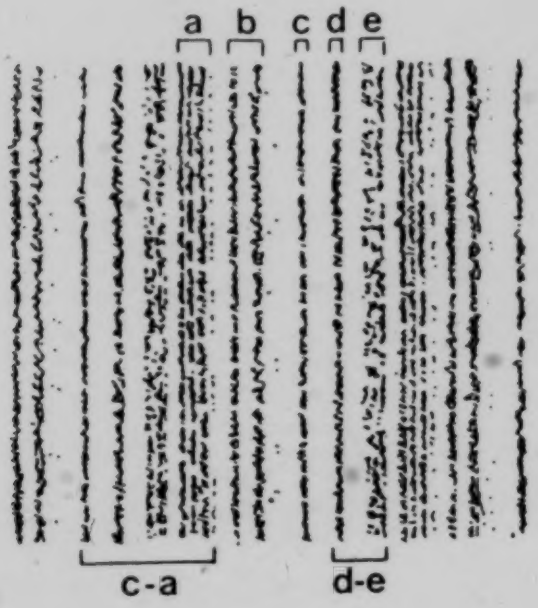

Fig. 21. Diagrammatic representation of a positively stained collagen fibril showing the name and location of the five major bands and their respective interband spaces that occur in each D period. Selected regions of the D period are also shown. Adapted from Bruns and Gross (1974).

TABLE 8
DISTRIBUTION OF FERRITIN PARTICLES AMONG COLLAGEN BANDS

Bands and Interband Spaces (-)	Percentage of Single Ferritin Particles Localized in Bands and Interband Spaces			Bands Only
	with 6D6[1]	with 7B1[1]	average 6D6 & 7B1	average 6D6 & 7B1[2]
a	9.5	14	12	18
-	1	2	1.5	
b	5	8.5	7	10
-	4	6	5	
c	15.5	13	14	20.6
-	10.5	5	8	
d	14	17	15.5	24
-	10	8	9	
e	20	15	17.5	27.5
-	10	12	11	

[1]Percentage values represent the average of triplicate analyses of electron photomicrographs produced using each MAb. An average of 91 ferritin particles (range 93 89) were counted on photomicrographs of fibrils incubated with 6D6; an average of 129 ferritin particles (range 125-134) were counted on photomicrographs of fibrils incubated with 7B1.

[2]The percentage of particles assigned to the bands only was derived by adding the average values for each band and half of the average value for the two adjacent interband spaces.

[3]The experimental protocol was designed to minimize the binding of normal mouse serum and ferritin conjugated antibodies to the collagen fibrils. The controls were negative.

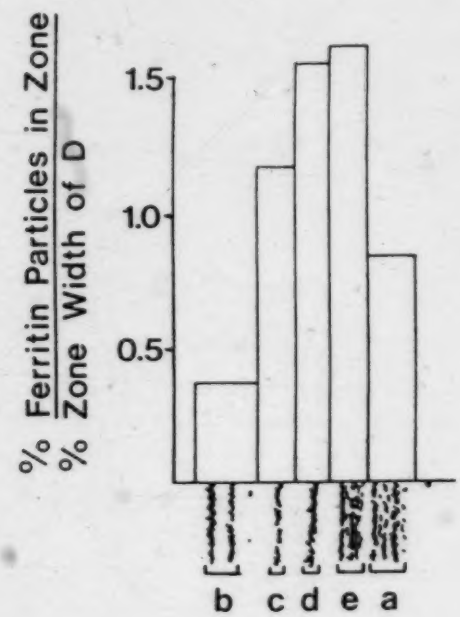

Fig. 22. Distribution of ferritin particles, by zone in a single D period of a collagen fibril. Each zone refers to the portion of the D period spanning a single band and one half the width of each adjacent interband space. The percentage of ferritin particles in each zone is derived from the data in Table 8.

S.D.

4. DISCUSSION

The major goal of this project was to develop several different monoclonal antibodies to PDS in order to monitor its tissue distribution at various stages of development, to evaluate its structural relationship with other proteoglycans and to study the structural basis of its biological activity such as collagen fibril binding. In particular, antibodies were sought to its protein determinants which, as discussed earlier, would make the MAbs more specific probes for this individual proteoglycan and certainly more useful in evaluating the role of its protein core. As a consequence, an experimental protocol was sought that would enhance the immunogenicity of these particular determinants.

The standard protocol for the production of monoclonal antibodies involves the use of Balb/c mice at all stages; immunization, fusion and ascites production. This is largely because all of the mouse plasmacytoma cell lines in use are derived from Balb/c mice and it is generally held that better results are possible if histocompatibility is maintained throughout the procedure. A potential problem in confining oneself to this particular strain of inbred mice for the initial immunization is that they may be genetically incapable of providing a strong antibody response to the antigen; particularly if the antigen is weak. Significantly, the protocols previously employed to produce MAbs to proteoglycans (i.e., the cartilage proteoglycan) have involved immunization of Balb/c mice with a protein core preparation of the molecule, produced by treatment with hyaluronidase (Dorfman et al., 1980) or chondroitinase ABC (Caterson et al., 1982). Although not specifically mentioned in the literature, it seems possible that attempts to produce MAbs using the intact proteoglycan as antigen met with little success and that removal of the bulk of the chondroitin sulfate side chains was designed to increase its antigenicity. Although this approach to improving antigenicity is valid, its use has resulted in modified antigens whose related MAbs have been directed against the glycosaminoglycans of this proteoglycan (Caterson et al., 1982; Caterson et al., 1983; Couchman et al., 1984; Jenkins et al., 1981); particularly the artifactual determinants within the galactosaminoglycan 'stubs' produced by the enzymes (Caterson et al., 1985; Jenkins et al., 1981). It became apparent that these

carbohydrate determinants were highly antigenic in Balb/c mice and as immunization of them with similar protein core preparations of PDS would probably result in the production of MAbs directed against similar or the same artifactual determinants, there seemed to be little advantage in following the "established" protocol for proteoglycans. As a result, intact PDS was used as the immunogen but to ensure that it was sufficiently antigenic to induce a strong antibody response, thereby increasing the number of antigen specific cells available for fusion, a high responder mouse strain to this form of PDS was sought and used (i.e., SJL/J). It is clear in retrospect that such an analysis was worthwhile as low (C3H.SW/Sn), medium (CBA.CaJ) and high (SJL/J and A.CA/Sn) responder strains of mice to PDS were discovered. Although this analysis did not guarantee that these mice were responding to the protein determinants of PDS, with the lack of artifactual determinants present and with the anticipated low immunogenicity of the native dermatan sulfate side chains (due to their common structure in different mammalian species and the fact that dermis is not a privileged tissue immunologically), it was felt that a strong response to this form of PDS would probably be based on its protein determinants. In the case of SJL/J mice this seems to have been true (see below). In spite of the fact that the antibody response by Balb/c (H-2d) mice to intact PDS was unpredictable and may not have been poor they were not included in the immunogenetic analysis due to their relative inability to respond to the protein core of the cartilage proteoglycan. It is noteworthy that Sundar Raj et al. (1985) immunized Balb/c mice with intact proteokeratan sulfate and found that all 28 antibodies produced were directed against various structural features of the keratan sulfate side chains only. The finding that A.CA/Sn (H-2f) mice also responded well to PDS is an incentive to use these mice in future fusion experiments as they could conceivably generate MAbs with specificities to protein determinants that may be unobtainable from mice with the (H-2s) haplotype.

4.1 The Specificities of the Monoclonal Antibodies

As a library of MAbs to PDS was desirable, it was important to determine whether the 4 MAbs selected for further study possessed different specificities and recognized different epitopes on the proteoglycan. The results of the competitive binding ELISA (Fig. 2) indicated that the 4 MAbs recognized at least 3 different antigenic determinants on PDS; one by 3B3, one by 7B1 and at least one by 1XA and 6D6. Other findings tended to confirm these results. When the antigenicity of PDS that had been incubated for 24 hours at 65°C in buffer lacking papain was compared to that of untreated PDS (Fig. 8), the inhibition curves that resulted using 3B3 and 7B1 differed from one another and from those produced by 1XA and 6D6 which were very similar. This pattern was also evident by the manner in which the 4 MAbs recognized different components of the CNBr digest of PDS (Plate 8), the CNBr digest of the (HF)core of PDS (Plate 9) and the fragments of PDS produced by digestion with cathepsin D (Plates 6 & 7). It was also evident that the specificity of 3B3 and its epitope differed from those of the other MAbs by the fact that it alone recognized a tryptic peptide of PDS (Plate 6). Similarly, 7B1 differed from the other MAbs by its inability to recognize human PDS preparations (Plates 15 & 16) or stain human tissue immunohistochemically (Plate 36).

The competition between 1XA and 6D6 to bind PDS may be explained by one of the following situations: *i)* the MAbs are identical and recognize the same epitope or the MAbs differ in specificity and recognize *ii)* the same epitope, *iii)* overlapping epitopes or *iv)* adjacent epitopes. The conclusion that 1XA and 6D6 do not share the same specificity was based on the unique ability of 1XA to bind to a component isolated from chick sternal cartilage (Fig. 17; Plate 23); by the relative inability of 1XA to locate specifically PDS in fixed tissue sections (Table 7); and by the way in which 1XA and 6D6 differed in their recognition of the (ABC)cores of PDS (Fig. 7). In the latter case the observed change in the slope of the inhibition curve of 1XA was unique and probably represented a change in the nature of the antigenic determinant as a result of enzyme treatment (see below). Therefore while good evidence exists to indicate that 1XA and 6D6 differ in specificity, it has not been possible

however to determine how close the epitopes of 1XA and 6D6 are. Knowing their exact relationship to one another may have to await the more extensive proteolytic cleavage procedures associated with amino acid sequencing.

4.2 The Nature and Location of the Epitopes in PDS

4.2.1 The Epitopes of 1XA and 6D6

As discussed above, the epitopes of these MAbs are thought to be close to one another. When a number of the enzymatically or chemically produced fragments of PDS were analyzed by immunoblotting, in most instances 1XA and 6D6 recognized the same fragments. The smallest of these fragments, produced by digestion of PDS with cathepsin D, had an Mr=14,000 (Plate 7). How close their epitopes might be within this fragment is difficult to determine as the MAbs were not observed to recognize separate smaller fragments.

There is considerable evidence to suggest that the epitopes of 1XA and 6D6 are not located exclusively on the dermatan sulfate side chain(s) of PDS. Both MAbs were effectively inhibited by the (ABC)cores of PDS (Fig. 7) and both MAbs recognized the (BES)cores (Plate 3), the (HF)core (Plate 1) and the protein core produced by digestion of PDS with cathepsin C (Plate 5), all of which have had the O-linked dermatan sulfate side chain(s) entirely removed. Also, the peptides recognized by both MAbs (Plates 7 & 8) appeared as distinct bands on immunoblots rather than broad, diffuse bands characteristic of peptides with glycosaminoglycan attached (i.e., Plate 6, lane 8).

Recognition by both MAbs of the (BES)cores and (HF)core of PDS also provided evidence that their epitopes do not consist exclusively of O-linked oligosaccharide side chains. Recognition of the (HF)core and its CNBr peptides (Plate 9), previously shown to possess a reduced amount of N-acetylglucosamine and a greatly reduced amount of N-acetylgalactosamine (Pearson et al., 1983) also argues against the possibility that either epitope consists exclusively of N-linked carbohydrate side chains. This is corroborated by the

finding that both 1XA and 6D6 recognized the (ABC)core of a structurally similar proteoglycan produced by human gingival fibroblasts treated with tunicamycin (Plate 4).

While it may be concluded that the glycosaminoglycan side chain(s) of PDS do not appear to contribute structurally to the integrity of the epitope of either MAb, the same cannot be said for the smaller oligosaccharide side chains. Although the marked loss in antigenicity of PDS after proteolytic digestion with papain (Fig. 8) or trypsin indicates that protein is essential to the integrity of both epitopes and probably forms the bulk of each, because the immunoblots did not adequately reveal the degree to which the MAbs bound the (HF)core or the (BES)cores, one cannot rule out the possibility that O-linked or N-linked oligosaccharide may be a minor component of either epitope. An attempt was made to analyze the degree to which the MAbs recognized the (HF)core of PDS using ELISA inhibition techniques, but its pronounced insolubility in other than strongly denaturing solvents made such an analysis difficult and hard to interpret.

Other results allow one to speculate further on the nature of each epitope within PDS. The inability of 1XA to bind to PDS in formalin fixed tissue sections of bovine skin suggests that the aldehydic reagent may be reacting with lysine or arginine residues within the epitope of 1XA, amino acids that may not be present or antigenically important in the epitope of 6D6. Also, the increased effectiveness of PDS as an inhibitor of 1XA and 6D6 after it had been incubated in buffer (lacking papain) for 24 hours at 65°C (Fig. 8) suggests that heat denaturation and possibly fragmentation of the proteoglycan under these conditions exposed the epitope(s) of 1XA and 6D6 to a greater degree thereby increasing their affinity. It is pertinent that PDS was extracted and purified in strong denaturing solvents. While it is conceivable that both MAbs may have been produced against a denatured form of PDS and that both epitopes are recognized fully only when PDS is denatured, both MAbs are able immunohistochemically to locate (presumably) native PDS in frozen, unfixed sections of bovine skin. Therefore, the increased affinity of both MAbs for heat treated PDS suggests that a part of the protein core of this proteoglycan may exist in some form of secondary or tertiary structure (reducing

accessability to all of the epitope) when in non-denaturing solutions. It is also possible that prolonged incubation at 65°C may have separated any PDS molecules that had aggregated in solution by noncovalent forces, thereby exposing more of the determinants (see below).

4.2.2 The Epitope of 3B3

Like 1XA and 6D6, 3B3 was not inhibited by 5000 ng/ml of dermatan sulfate and did recognize the (BES)cores, the (HF)core and the cathepsin C-produced protein cores of PDS, all of which suggest that its epitope does not involve the dermatan sulfate side chain(s) of PDS. The CNBr fragments of PDS recognized by 3B3 (Plate 8) were also distinct bands on immunoblots and not diffuse, broad bands characteristic of glycosaminoglycan-containing peptides. However, like the other 2 MAbs, the involvement of the N-linked or O-linked oligosaccharides in its epitope cannot be ruled out, in spite of the finding that papain digestion caused a loss in antigenicity of the proteoglycan (Fig. 8). It was not determined whether the small tryptic peptide recognized by 3B3 also stained with PAS.

It is apparent that 3B3 did not recognize the major CNBr peptides transferred to nitrocellulose, but others presumed to be present in small quantities of which several at least are probably the result of incomplete cleavage by CNBr (Plate 8). Recognition of a pair of peptides slightly larger than the 2 major CNBr peptides stained by Amido black suggests that the epitope of 3B3 may be within a small peptide (Mr=3,000-5,000) adjacent to them, possibly represented by the peptide stained by 3B3 at the dye front (there is evidence, discussed below, that the two large CNBr peptides stained by Amido black differ only in the number of attached oligosaccharide side chains). Alternatively, the epitope of 3B3 may include amino acids from both peptides. The decreased effectiveness of PDS as an inhibitor of 3B3 after it had been incubated in buffer containing cysteine HCl for 24 hours at 65°C also suggests that the integrity of its epitope is influenced by the arrangement of disulfide bonds in PDS. Although this proteoglycan contains a significant number of half-cystine residues (Table 4), the number and position of any intramolecular disulfide bonds remains unknown. However, it seems likely that

under the conditions of incubation, the cysteine HCl may have induced an abnormal arrangement of disulfide bonds within and/or between PDS molecules through disulfide interchange thereby obscuring some of the epitopes of 3B3.

4.2.3 The Epitope of 7B1

While 7B1 was also inhibited by the various protein core preparations and was not inhibited by dermatan sulfate, it became apparent on immunoblots (Plates 6 & 7) that its epitope was near the site at which a dermatan sulfate side chain is attached and may reside within the NH_2-terminal CNBr peptide. This was confirmed by nitrocellulose electrophoresis and subsequent immunochemical staining of a preparation consisting largely of CB-1 (Table VI) which revealed that 7B1 recognized the glycosaminoglycan-bearing CNBr peptide and that the peptide possessed virtually the same mobility as the dermatan sulfate standard (Plate 10). Nitrocellulose electrophoresis of the insoluble fraction of a CNBr digest of PDS and subsequent immunochemical staining revealed that 7B1 was the only MAb to recognize this component (Plate 11). The other MAbs recognized protein-rich fragments that had adsorbed to the nitrocellulose and did not migrate. Likewise, 7B1 recognized components at the origin, such as the Mr=45,000 fragment (Plate 7), that had probably arisen by incomplete cleavage and, possessing more peptide, had adsorbed to the nitrocellulose and not migrated.

The results of the immunoblots of the various protein core preparations discussed above together with the loss in antigenicity of PDS after digestion with papain (Fig. 8) indicated, like the other MAbs, that the epitope of 7B1 consists largely of protein. The additional finding that 7B1 did not recognize human skin PDS (Plate 16) or immunochemically stain sections of human tissue (Table 7) suggested that if human PDS possesses a structure homologous to bovine PDS, some portion of its NH_2-terminal amino acid sequence should differ from that reported for bovine skin PDS (Pearson et al., 1983). Recently, Brennan et al. (1984) sequenced the NH_2-terminal of the protein core of a proteoglycan isolated from human fetal membranes. The sequence of the first 9 residues was identical to that of bovine skin PDS, including the likelihood

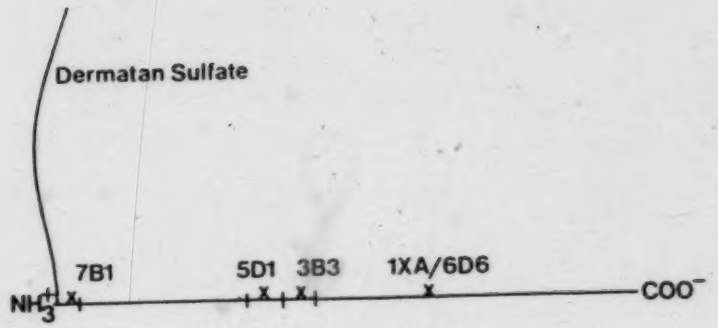

Fig. 23. Schematic representation of bovine skin PDS with its protein core in extended conformation to show the tentative locations (x) of the epitopes of the MAbs, including that of 5D1. The vertical lines represent methionine residues that delimit CNBr peptides. (Based on the methionine content of PDS other, smaller CNBr peptides are also believed to be present). Other than 7B1, the location of the epitopes within each of the peptides is unknown, except that the epitope(s) of 1XA/6D6 are at least Mr=5,000 from the C-terminal of the large CNBr peptide.

of a carbohydrate substitution at residue 4. However, except for a shared glutamic acid residue at position 17, the amino acid sequence from residues 10 to 23 differed. An affinity purified antiserum to this human proteoglycan immunoprecipitated a PDS (Mr=120,000) present in culture medium from IMR-90 human embryonic lung fibroblasts that possessed 2 (ABC)cores with the same apparent molecular weights as those of the fetal membrane proteoglycan (Brennan et al., 1984). Based on the presumption that the two human proteoglycans share the same protein core(s), the possibility arises that the epitope of 7B1 resides in the amino acid sequence of bovine skin PDS between residues 10 and 21, a portion of the sequence not as highly conserved as that of the first 9 residues, which may function as a recognition site for xylosyl transferase.

It may be concluded therefore that the different epitopes consist largely, if not exclusively, of protein and that any contribution from carbohydrate is due to the proximity of N-linked or O-linked oligosaccharides. Furthermore, in spite of the fact that PDS was extracted with denaturing solvents and may not have been wholly native, none of the MAbs required that PDS be fully denatured to be recognized (Table 7). However, recognition by the MAbs on immunoblots of various peptide fragments of PDS that had been boiled in the presence of SDS, urea and 2-mercaptoethanol also indicates that none of the MAbs required that PDS be completely native to be recognized. These observations suggest that the epitopes of the 4 MAbs are more likely to be sequential in nature rather than conformational (i.e., relying on tertiary structure). Also, on the basis of shared recognition of peptides, such as breakdown products in PDS preparations, incompletely cleaved CNBr peptides and the fragments produced by digestion with cathepsin D, it became apparent that the epitope of 3B3 is situated between those of 7B1 and 1XA/6D6.

4.3 The Structure of Bovine Skin PDS

Just as the various fragments of PDS have helped to determine something of the nature and approximate location of each epitope, the MAbs in turn, by their sensitivity and specificity in recognizing PDS and its various fragments, have been able to shed additional light on the structure of the proteoglycan itself.

Perhaps one of the most significant early findings was that a PDS from mature bovine skin may possess one of two different (ABC)cores. Although not easily seen when electrophoresed on 10% (or higher concentration) SDS-polyacrylamide gels nor when large loads of sample were applied, they became readily discernible on immunoblots when lower amounts of sample were applied to more porous gels (i.e., Plate 1, lane 12). The finding that only a single (HF)core was produced and was recognized by all 4 MAbs suggested that the 2 (ABC)cores did not differ in their protein content, but in the extent to which they were substituted with carbohydrate. The observation that two similar protein cores were detected after digestion of

PDS with cathepsin C and after β-elimination sulfite addition indicated that the observed difference in apparent molecular weight between these other protein cores and the (HF)core was due almost exclusively to the N-linked oligosaccharide side chains. Furthermore, the observed difference in apparent molecular weight between the (ABC)cores themselves (Mr=55,000 and Mr=50,000) was due to the likelihood that 80-90% of the protein cores of mature bovine skin PDS contained one or more additional N-linked oligosaccharides. The large pair of CNBr peptides of bovine skin PDS stained by Amido black and PAS are probably derived from their respective (ABC)cores and as their relative mobilities decreased dramatically after treatment with anhydrous HF (Plate 9) they probably also differ in their content of N-linked oligosaccharide.

More definite evidence on this question was obtained by the study of an antigenically related proteoglycan shown by the MAbs to be present in culture fluid from human gingival fibroblasts. Likely a PDS (see below), this proteoglycan also possessed 2 (ABC)cores, but they were present in more equal proportions (Glössl et al., 1983). When these fibroblasts were incubated in medium containing tunicamycin only a single (ABC)core (Mr=43,000) resulted confirming the role of the N-linked oligosaccharides in the formation of the 2 (ABC)cores. These results also suggest that bovine skin and human gingival PDS protein cores are very similar in size and that a tissue and/or species difference may exist in the degree to which the protein core is glycosylated with these oligosaccharides.

The MAbs have also revealed several other aspects of the structure of PDS. The isolation and characterization of CB-1, together with the manner in which it and other glycosaminoglycan-bearing peptides were stained on immunoblots by 7B1, helped to confirm that the moiety attached to residue 4 in bovine skin PDS is a dermatan sulfate side chain. Recognition by the MAbs of the protein core produced by digestion with cathepsin C also helped as well to confirm that a large majority, if not all, bovine skin PDS molecules possess a single dermatan sulfate side chain. The lack of recognition by 1XA or 6D6 of any CNBr peptides smaller than the large pair stained by Amido black helped to confirm that these peptides,

representing about one half of the protein core, are individual peptides and not the result of incomplete digestion by CNBr. Also, the finding that the epitopes of 7B1 and 3B3 appear to be separated from one another by an intervening CNBr peptide with Mr=12,000-14,000 and that both are closer to the NH_2-terminal of the protein core of PDS than are those of 1XA and 6D6 suggests that the large CNBr peptides, differing in N-linked oligosaccharide content, are located near, or represent, the C-terminal portion of the molecule.

It was also found that the MAbs recognized a component on immunoblots of bovine skin PDS preparations (Plates 9, 12, 13 and 19) that appeared as a broad, diffuse band with an apparent molecular size approximately twice that of PDS (Plate 19). Although present in small quantities, this component was of interest as it was possible, based on the shared epitopes, that it represented a precursor form of PDS or a proteoglycan with a similar protein core possessing more and/or longer glycosaminoglycan side chains. This component was not observed in the various protein core preparations of PDS. Instead a component, or components, with an apparent molecular size approximately twice that of the respective protein core in each preparation was observed (Plates 1-3). In the preparations of the (ABC)cores and the (BES)cores, it had an Mr=92,000-98,000. In the preparation of the (HF)core it had an Mr=76,000. In all cases, it resembled the protein core more than the intact proteoglycan as the band was not broad and diffuse. Its appearance together with the loss of the larger diffuse band after enzymic or chemical treatment of PDS suggested that the smaller component may be derived from the larger (i.e., its protein core).

Immunoblots of samples from each peak of a Sephacryl S-300 chromatograph of an (ABC)core preparation of bovine skin PDS (Fig. 20) indicated that multimers of the (ABC)core could arise by forming intermolecular disulfide bonds (Plate 26). The development of such multimers, following the use of buffers containing 4M GdnHCl, has been reported previously (Oike et al., 1980; Noro et al., 1983). Equivalent multimers of an antigenically related proteoglycan were present in the non-reduced sample of bovine A1D4 (Plate 22), a

fraction prepared by CsCl density gradient centrifugation in the presence of 4M GdnHCl. It was of interest that a disulfide bonded multimer of the (ABC)core, most probably a dimer, migrated with Mr=92,000-98,000. However, it was also apparent that small quantities of a component migrating as a dimer had resisted the standard reduction conditions used (Plate 26) and probably represented the same component referred to above. The relative proportion of this component in an (ABC)core preparation was not significantly decreased by incubation in sample buffer containing 10% 2-mercaptoethanol for 6 hours at 70°C (results not shown), but observably decreased when it was boiled for 4 minutes in sample buffer containing 10% 2-mercaptoethanol, 8M urea and 10% SDS (Plate 27), suggesting that its size may be due to the presence of very resistant disulfide bonds and/or to strong noncovalent interactions between smaller molecules. When the higher molecular weight proteoglycan fractions (peak 1) from skin (results not shown) and dental pulp (Plate 25) were analyzed on immunoblots numerous antigenically related components of variable size and relatively resistant to reduction were also detected both before and after the fractions had been digested with chondroitinase ABC. Components with sizes equal to those of several PDSs and their (ABC)cores (see below) were present, but it was apparent that even after treatment with chondroitinase ABC a single (ABC)core did not result. Although the nature of these antigenically related components remains to be determined, the weight of evidence suggests that they were all multimers of PDS. Dimers of PDS may have arisen by the formation of reduction-resistant bonds between their protein cores. The persistence of multimers on immunoblots of the protein core preparations of PDS suggests that strong noncovalent bonds and/or disulfide bonds very resistant to reduction, between the protein cores themselves, are important in their stabilization. It also remains to be determined whether these dimers of the protein core of PDS arise as a byproduct of the enzymic or chemical processes used. The difference in the nature of the antigenic determinants recognized by 1XA in preparations of the (ABC)cores of PDS may have been due to the presence of aggregates or multimers in the solution. Likewise, heat denaturation may have separated such aggregates increasing the affinity of 1XA.

The origin of the "multimers" present in the higher molecular weight proteoglycan fractions of skin and pulp is of interest as, in spite of the possibility that they may have been formed artifactually during isolation and purification, it is conceivable that they exist *in vivo* and have been extracted as such. It is also possible that some of the "multimers" in peak 1 may consist of PDS bound to other connective tissue components (i.e., collagen αchains).

In conclusion, all available data regarding the structure of bovine skin PDS, including that discussed above, indicate that it consists of a protein core (Mr=43,000) to which are typically attached a single dermatan sulfate side chain (molecular weight = 16,000-17,000), a variable number of N-linked oligosaccharides and probably several small O-linked oligosaccharides (Nakamura et al., 1983). The N-linked oligosaccharides occur in definite numbers on the large CNBr peptide which is located at or near the C-terminal of the protein core. The occurrence of half-cystine residues in PDS suggests the existence of intramolecular disulfide bonds although the lack of a noticeable change in its apparent molecular weight with reduction suggests that they do not bridge large portions of the primary structure. Intermolecular disulfide bonds can form, but may not *in vivo*. The protein core of PDS has a tendency to self-associate although the significance of this *in vivo* is unknown. Finally, the immunological data provided by 1XA and 6D6 hints at the possibility that the protein core of this proteoglycan may normally possess a significant amount of tertiary structure.

4.4 The Nature of Antigenically Related Proteoglycans

Bovine periodontal ligament proteodermatan sulfate has been isolated and characterized (Pearson & Gibson, 1982). Its amino acid composition is very similar to that of bovine skin PDS (Table 4) but it was shown to be a larger proteoglycan consisting of longer (molecular weight=29,000 daltons) L-iduronic acid-rich dermatan sulfate side chains (Pearson and Gibson, 1982). All 4 MAbs recognized this proteoglycan (Mr=116,000) and its (ABC)core (Mr=54,000) and confirmed that the protein core of this proteoglycan is similar to that of skin

PDS. The results also indicated that periodontal ligament PDS is larger than mature skin PDS due to its dermatan sulfate side chains. It remains to be determined whether periodontal ligament PDS contains a dermatan sulfate side chain attached to residue 4 but, based on the finding that the observed difference in the apparent molecular weights of skin PDS and its (ABC)cores on SDS-polyacrylamide gels (about 30,000) is due to the presence of a single side chain with an average molecular weight of 16,000 daltons, it seems possible that the larger size of periodontal ligament PDS on SDS-polyacrylamide gels (about 25,000) may be due to the presence of a single dermatan sulfate side chain with an average molecular weight of 29,000 daltons.

The 4 MAbs also recognized the proteoglycan (Mr=116,000) and its (ABC)core (Mr=54,000) present in the PG2 fraction of bovine gingiva. The similarity to bovine periodontal ligament PDS is obvious and suggests that this proteoglycan is a PDS of the same structure. This is consistent with the finding that L-iduronic acid-rich dermatan sulfate was the only glycosaminoglycan found in this fraction and that the amino acid composition of the sample was similar to that reported for bovine skin and periodontal ligament PDS (Pearson & Pringle, manuscript submitted). A proteoglycan (average Mr=120,000) in the human gingiva PG2 fraction (Plate 15) was also recognized by 1XA, 3B3 and 6D6 and based on the content of L-iduronic acid-rich dermatan sulfate in the sample (Pearson and Lehocky, unpublished results) was thought to be a PDS. Its (ABC)cores possessed mobilities similar to those of bovine skin PDS, but were present in more equal proportions. The same proteoglycan is presumed to have been recognized by 1XA, 3B3 and 6D6 in the concentrated culture medium of human gingival fibroblasts (Plate 17). The relative amounts of the (ABC)cores are consistent with those of a PDS synthesized by human skin fibroblasts (Glössl et al., 1983) and suggests a species dependence in the degree of glycosylation. Like periodontal ligament PDS, the larger size of human gingiva PDS appears to be due to the presence of longer dermatan sulfate side chain(s) reported to have an average molecular weight of 25,000-27,000 daltons (Bartold et al., 1982). Evidence to suggest that there may be only one dermatan sulfate side chain has come

from the recent finding that digestion of human gingival PDS with the exodipeptidase cathepsin C resulted in protein cores lacking a glycosaminoglycan side chain (Pearson & Lehocky, unpublished results).

A proteodermatan sulfate was also isolated from fetal calf skin and studied on immunoblots with the 4 MAbs. It appeared to possess 2 (ABC)cores with the same apparent molecular weights as those of PDS present in mature skin, although the smaller form was barely detectable. Fujii and Nagai (1980) and Nakamura et al.(1983) reported that calf skin PDS possessed a single (ABC)core with Mr=55,000. However, the overall apparent size of fetal calf skin PDS (Mr=90,000) was larger than that of adult skin PDS (Mr=78,000) and once again the results indicated that the difference was due to a greater content of glycosaminoglycan. Because the dermatan sulfate side chains of fetal skin PDS co-migrated with those of adult skin PDS on cellulose acetate at pH 3.5 and in 0.1N HCl (results not shown), but migrated more slowly (Mr=34,000) than the adult dermatan sulfate side chains (Mr=26,000) in a 10% SDS-polyacrylamide gel, it was concluded that the larger apparent size of fetal calf skin PDS was due to the presence of longer dermatan sulfate side chain(s). The presence of a single dermatan sulfate side chain in calf skin PDS is consistent with the conclusions of Nakamura et al. (1983) although they reported its Mr=17,500, similar to the finding of Pearson & Gibson (1982) for adult skin dermatan sulfate side chains. The discrepancy could be due to the fact that their tissue sample was more developmentally mature (i.e., newborn) than the sample used in this study. Fujii and Nagai (1980) also reported that calf skin PDS side chains had an Mr=17,000, although they estimated that 3-4 of these side chains were present. The finding in the present study that the glycosaminoglycan side chains of skin PDS possibly decrease in length as the tissue matures is not unprecedented; the chondroitin sulfate side chains of the cartilage-specific proteoglycan have also been reported to decrease in size with age (Fellini et al., 1981).

The PG2 fraction from bovine dental pulp has been shown (Pearson & Pringle, manuscript submitted) to contain chondroitin sulfate-like (85%) and L-iduronic acid-rich

dermatan sulfate (15%) side chains. This result suggested that the fraction may have consisted of two distinct proteoglycans; a typical PDS and a chondroitin sulfate-containing proteoglycan. An Amido black stained blot of dental pulp PG2 (Plate 24) indicated that its major constituent was a proteoglycan (Mr=135,000) consisting of an (ABC)core with an Mr=100,000. The 4 MAbs however, recognized a minor constituent (Mr=116,000) and its (ABC)core (Mr=54,000) that were similar in size to the PDS and its (ABC)core present in periodontal ligament and gingiva. ELISA inhibition studies with the 4 MAbs revealed that bovine pulp PG2 was slightly inhibitory and suggested that if the inhibitor was PDS, it represented about 6% of the protein in the sample [6.5% (3B3), 6.5% (6D6) and 5% (7B1)]. Based on the content of L-iduronic acid-rich dermatan sulfate in the sample and the immunological results, it seemed possible that PDS represented a minor constituent of pulp PG2. The relatively low amount of immunohistochemical stain present in unerupted bovine dental pulp (Plate 35) also suggested that PDS was not a major constituent of this tissue. The major proteoglycan present in bovine dental pulp PG2 contained a protein core antigenically dissimilar to that of PDS and presumably one or more chondroitin sulfate-like side chains. The MAbs also recognized PDS and its "multimeric" forms on immunoblots of pulp PG1, suggesting that PDS was a minor constituent of this proteoglycan fraction. This is consistent with the prediction from the ELISA inhibition studies that less than 5% of the protein of the sample was PDS.

To summarize, the results of these studies indicated that fetal calf skin, gingiva and dental pulp, like mature skin and periodontal ligament, contained PDS. It also appears that PDS may have a characteristic structure, consisting of a single dermatan sulfate side chain with a molecular weight ranging from 16,000 to 29,000 daltons attached to a protein core with an Mr=43,000. The size of the glycosaminoglycan side chain and the number of N-linked oligosaccharides attached to the protein core appears to vary with the species, cell or tissue source and stage of differentiation/development. This notion is consistent with the results of others regarding the structure of PDS (see Table 3), including the more recently characterized PDS described by Brennan et al. (1984). It is of interest that only the PDS from mature human

skin may possess dermatan sulfate side chains with the same apparent size as those of mature bovine skin (Plate 16).

Antigenically related proteoglycans, of very similar size, were also discovered to be minor constituents of the high buoyant density (A1D1) and low buoyant density (A1D4) fractions isolated from bovine nasal cartilage (Plates 20 & 22). The apparent molecular weights of the intact proteoglycans and their (ABC)core(s) were very similar to those of periodontal ligament PDS and their recognition by all 4 MAbs suggests that their protein core is the same as that of the other PDS proteoglycans. However, the nature of their glycosaminoglycan side chain(s) is unknown. These proteoglycans in the two fractions probably represent the same molecule; its relative abundance in the A1D4 fraction (2-3% of the sample protein by ELISA inhibition vs <0.5% in A1D1) is probably due to its high protein content. The inability of the MAbs to detect a similar proteoglycan in rat chondrosarcoma fractions is probably due to the similarity between rat and mouse antigens.

The origin of this proteoglycan in the bovine cartilage fractions is of interest. Cartilage has been shown to contain several proteoglycans smaller than the cartilage-specific monomer, particularly in developing cartilage matrices (Goetinck et al., 1974; Okayama et al., 1976; Noro et al., 1983; Shinkai et al., 1983). A small proteoglycan (Mr=90,000) possessing 2 (ABC)cores (Mr=48,000 and Mr=45,000), L-iduronic acid-poor (< 40%) dermatan sulfate and N-linked oligosaccharide side chains was isolated from bovine fetal epiphyseal cartilage (Rosenberg et al., 1982). A rabbit antiserum to this proteoglycan crossreacted immunohistochemically with the same or a similar antigen in dermis, in the stratum germinativum of the epidermis and in the matrix of adult articular cartilage. It may represent the proteoglycan found by the MAbs in the A1D1 and A1D4 fractions of bovine nasal cartilage. However, the finding that none of the MAbs stained the matrix of mature bovine nasal cartilage (Plate 36a) or the stratum germinativum of the epidermis (Plates 28b, 29c, 31 and 32) suggests that the proteoglycan isolated by Rosenberg et al. (1982) may have a protein core that differs from that of PDS and/or the MAbs are more specific for PDS than the antiserum. It is possible therefore that the

proteoglycan recognized by the MAbs in the cartilage fractions may represent contamination by a PDS from the investing layers of connective tissue (Plate 36a) and may not be a constituent of the matrix at all.

3B3 and 6D6 (and possibly 1XA) also detected a proteoglycan (Mr=135,000) in concentrated culture fluid of monkey arterial smooth muscle cells (Plate 18). Its (ABC)core had an Mr=43,000 (Plate 18), the same as the (HF)core of PDS (Plate 1) and the (ABC)core of PDS from gingival fibroblasts treated with tunicamycin (Plate 4). Chemical characterization of the proteoglycans in the culture fluid of these cells (Chang et al., 1983) indicated that a proteoglycan with L-iduronic acid-rich dermatan sulfate side chains was not present in significant amounts. While it is certainly possible that the more sensitive immunoblotting method has detected a PDS (Kapoor et al., 1981) that the other methods have overlooked, it appears that the molecule the MAbs have recognized most likely represents a small proteoglycan that possesses one or more L-iduronic acid-poor dermatan sulfate side chains (Mr=43,000), no O-linked oligosaccharides and a few N-linked oligosaccharides (Chang et al., 1983). Recognition of the (ABC)core of this proteoglycan by 3B3 and 6D6 suggests that it possesses a similar, or the same, protein core as PDS. If the protein cores are the same, then this protein core may also possess a single L-iduronic acid-poor dermatan sulfate side chain. This is not an unreasonable possibility as this proteoglycan would then differ from PDS only in the degree to which the D-glucuronic acid residues of the original chondroitin sulfate side chain were epimerized. This possibility is also corroborated by the recent results of Brennan et al. (1984) who found that an affinity purified antiserum to a small proteoglycan bearing L-iduronic acid-poor dermatan sulfate side chain(s) immunoprecipitated a PDS with the same 2 (ABC)cores (Mr=45,000 and Mr=43,000). The possibility that at least 2 of the MAbs may be crossreacting with a proteoglycan possessing a protein core that differs considerably from that of PDS seems unlikely and therefore, very significantly, these results imply that the MAbs are not necessarily specific for PDS; only for its protein core which may have a structurally different galactosaminoglycan attached to it. None of the MAbs crossreacted with the cartilage

specific proteoglycan monomer (Fig. 14; Plate 20) or the major proteoglycans containing chondroitin sulfate present in pulp PG1 (Fig. 19; Plate 25) and pulp PG2 (Fig. 18; Plate 24).

4.5 Immunohistochemical Localization of the Protein Core of PDS

All 4 MAbs were used in conjunction with immunohistochemical techniques in order to locate PDS. All of the tissues examined immunohistochemically in this study, with the exception of bovine nasal cartilage, have been shown to contain PDS. However, with the discovery that at least several of the antibodies may crossreact with the protein core of a proteoglycan bearing L-iduronic acid-poor dermatan sulfate side chains, some degree of caution must be exercised in the interpretation of these results. Strictly speaking the MAbs may be locating the protein core of either proteoglycan in any of these tissues. The demonstrated lack of reactivity of the MAbs with other components such as collagen, fibronectin, elastin, albumin, the link proteins and several other proteoglycans also helps to confirm the specificity of the MAbs for the protein core when used immunohistochemically.

All 4 MAbs stained the same areas of adult bovine skin sections (Plate 28a) although the degree of staining by each varied with fixation (Table 7). The pattern of the stain, present throughout the dermis and closely associated with the many collagen fibers, resembled the pattern of immunofluorescence obtained by Caterson et al. (1982) on sections of rat skin using an antiserum specific for the galactosaminoglycan stubs remaining after chondroitinase ABC digestion The stain was noticeably heavier in the superficial regions of the dermis where the collagen fibers were much finer. This was also evident in sections of human skin (Plate 31), bovine gingiva (Plate 32) and the investing layer of bovine tail tendon (Plate 34). Although the presence of the antigen in the interfibrillar spaces of some of these tissues cannot be ruled out, the pattern of staining is consistent with the notion that the molecule binds to the surface of collagen fibrils (Scott, 1980; Scott, 1984) in that much more of the antigen would be available for the MAbs to bind on the external surface of many small fibers in an area than on the surface of several large fiber bundles (often cut obliquely) in the deeper dermis. This contrast was

particularly evident in sections of bovine tail tendon cut longitudinally. Staining within the large fiber bundles, particularly within the deeper dermis, is presumed to represent exposed antigen surrounding the constituent fibers and fibrils. While it is difficult to judge at the light microscopic level whether the MAbs have recognized components within the various basement membranes, the lack of stain within the epithelial structures such as hair follicles, sweat and sebacceous glands and ducts, nasal glandular tissue, epidermis or the odontoblastic cell layer in dental pulp confirms the functional association between PDS and interstitial collagen fibers.

Of considerable interest was the observation that fetal calf skin, estimated to be about 130 days old (Lyne and Heideman, 1959) stained differently from adult bovine skin (Plate 29a). In fetal skin the stain was largely confined to the deeper layer of the dermis although when the enzyme reaction was prolonged with 6D6 a fine loose reticulum of fibers was observable within the superficial dermis amongst the developing hair follicles. During the embryological development of skin, there is a transition in the proportions of its constituent glycosaminoglycans. Hyaluronic acid and, in particular, chondroitin sulfate decrease and L-iduronic acid-rich dermatan sulfate increases (Breen et al., 1970; Nakamura and Nagai, 1980; Parry et al., 1982; Scott et al., 1981) presumably in the form of a PDS described above and by others (Fujii and Nagai, 1980; Nakamura et al., 1983). This increased deposition of dermatan sulfate which is simultaneous with the increase in the deposition of collagen (Nakamura and Nagai, 1980) has led to numerous suggestions that PDS somehow plays a critical role in controlling collagen fibril diameter (Parry et al., 1982 and others). It is apparent by the manner in which the fetal dermis has stained that PDS and collagen are closely associated with one another; even within the very fine (presumably) collagenous reticulum in the superficial layer of the dermis. The gel-like appearance of the embryonic superficial dermal connective tissue matrix suggests that it contains a relatively large amount of hyaluronic acid and a chondroitin sulfate-containing proteoglycan at this stage.

Differentiation within the dermis of a calf necessarily involves the development of hair follicles. At about 130 days gestation the follicles are not surrounded by much dense collagenous

matrix (Plate 29a), but as they continue to develop, their bases begin to become surrounded by such a matrix (Plate 30a). It is possible that rather than continuing to penetrate deeper into the dermis, the developing follicles are lengthened by an outward and interstitial growth of (dense) connective tissue. Such growth and the cell migration necessary for the differentiation of the sweat and sebaceous glands and arrector pili muscle associated with each follicle, may require that hyaluronic acid and the putative chondroitin sulfate-bearing proteoglycan remain in the superficial layer of the dermis for the period of time necessary to complete follicle development. The pattern of staining in a section of more mature fetal dermis suggests that this might be the case (Plate 30b). While PDS and a collagenous matrix continue to become more prominent between the follicles in the deeper dermis and a subepithelial zone of collagen and PDS becomes increasingly prominent, there is an intermediate zone, where the proposed differentiation is continuing, that remains relatively free of PDS and, presumably, dense collagenous matrix (Plates 30b and 30c). Ultimately, this zone disappears from the fetal dermis and the skin begins to look more like that of an adult (Plate 30d).

The ability of at least one MAb to locate the protein core of PDS in the fine reticulum of the superficial dermis of fetal calf skin and all of the MAbs to stain the fine collagenous matrix (endotendineum internum) investing tendon fibers (Plate 34) and the fine fibrils of unerupted bovine dental pulp (Plate 35) suggests that the protein core may bind to type III collagen fibrils as well as to those of type I.

4.5.1 Ultrastructural Localization of the Protein Core of PDS on Tendon Collagen Fibrils

As had been discussed earlier, considerable evidence exists to suggest that PDS and type I collagen fibrils are physically associated with one another, and not surprisingly, the suggested functions of PDS are based on this close association. A substantial amount of data suggests that PDS may somehow play a critical role in the fibrillogenesis of type I collagen with the most recent report (Vogel et al., 1984) demonstrating that PDS retards the formation of collagen fibrils *in vitro*. Scott and Orford (1980), based on the use of cationic dyes, have proposed that

the protein core of PDS is located at the *d* band of the D period, in the gap zone. As a result, they have suggested that PDS may inhibit the radial growth of collagen fibrils by restricting the access of collagen subassemblies to crosslinking sites within the gap zone. They have also suggested that PDS, with its high charge and large excluded volume, would retard or inhibit collagen fibril calcification within the holes of the gap zone (Scott and Orford, 1980; Scott, 1984). Thus it is apparent the physiological significance of this proteoglycan is very likely based on its position within the D period of collagen fibrils. Scott and coworkers (Scott, 1980; Scott et al., 1981; Scott and Orford, 1981) have attempted to locate it but as pointed out earlier their methods lack specificity and their interpretations are open to question. By virtue of their specificity, 2 different MAbs (6D6 and 7B1) were used immunohistochemically to locate the protein core of PDS on bovine tail tendon collagen fibrils. Samples of skin were initially used for these studies, but the irregular orientation of their constituent collagen fibrils made it difficult to observe fibrils oriented longitudinally.

Use of the peroxidase anti-peroxidase method necessarily involved treating the tissue sample with OsO_4 which also decreased the effectiveness of uranyl acetate to highlight the collagen banding pattern. With the limited enzyme reaction time of 5 minutes, small particles of precipitate were formed, the majority of which were located on one side of the single dominant band stained by the OsO_4 (Plates 37a-c). While it was not possible to conclude where the protein core was bound within the D period, the rather regular arrangement of the particles on the surface of the fibrils suggested that the protein core was also related to collagen in a regular periodic fashion. In fact, the pattern observed was remarkably similar to that obtained by Ruggeri and Benazzo (1984) who stained tendon collagen fibrils with Alcian blue and showed that the stained component was D-periodic and located consistently on one side of the major band stained by OsO_4.

Use of the MAbs with the immunoferritin method increased the resolution for several reasons. First, as it could be used in conjunction with uranyl acetate it was possible to relate the electron dense ferritin particles to the 5 major positively stained bands within each collagen D

period. Second, the electron dense ferritin particles were much more uniform in size and smaller than the oxidized precipitate of diaminobenzidine making it easier to relate their position to the bands and interband spaces. Also, as it is likely that both 6D6 and 7B1 recognize only a single epitope on the protein core, only 1 MAb would be bound to each PDS molecule which would reduce to a minimum the number of ferritin particles associated with a single antigen. In fact, only single isolated ferritin particles were counted as they more accurately reflected the position of the protein core of a single PDS molecule.

The observed distribution (Fig. 22) indicates that the protein core is most probably attached to collagen fibrils within each D period in the vicinity of the *d* and *e* bands. The conclusion that the protein core binds to a specific region on the surface of the collagen fibrils implies that it has a specific "collagen fibril binding site" and that one of its functions is to position this molecule on the fibril surface in a specified manner. Like enzymes and antibodies, such a recognition function suggests that at least a part of the protein core possesses a globular tertiary structure and is consistent with previous conclusions based on immunological results and more recent studies analyzing the secondary structure of the protein core by circular dichroism (P.G. Scott, personal communication). Such a function would also explain why, on immunoblots, the protein cores of PDS preparations from various tissues possess a similar structure. Furthermore, the implied dependence of binding on the protein core is consistent with the result of Vogel et al. (1984) who found that the (ABC)core of PDS was almost as effective as the intact molecule in inhibiting fibrillogenesis of type I collagen molecules *in vitro*.

Although the distribution of the ferritin particles indicates only that the probable binding site on the surface of collagen fibrils is in the vicinity of the *d* and *e* bands, it seems reasonable to speculate that it might be between these bands. Not only is such a location nearer the symmetrical center of the observed distribution than either band, it is also a region containing a substantial number of hydrophobic residues (and thus does not stain with uranyl acetate) and would therefore permit protein-protein interactions involving hydrophobic as well as charged residues. Furthermore, as the bands are dominated by charged residues, their

tendency to resemble one another decreases their potential as specific binding sites.

The present data suggest therefore that PDS molecules, via their protein cores, surround the circumference of collagen fibrils like a beaded necklace clasping the *d-e* interband space in each D period. The dermatan sulfate side chains may lie adjacent to the collagen fibril and/or project radially, but not necessarily rigidly, away from its surface forming a polyanionic microenvironment around each fibril. How densely packed the PDS molecules are in each D period is unknown.

These results and the conclusions drawn from them differ somewhat from those of Scott (1980) who contends that collapsed glycosaminoglycans have highlighted the protein core located at the *d* band. It seems possible that during dehydration the dye-bound glycosaminoglycans may collapse against the collagen fibril and lie along side the beaded necklace of protein cores and that of the "sides" available the one nearest the charged *d* band may be more attractive to the negatively charged glycosaminoglycans. The preference for the *d* band may also be due to the location of residue 4 within the structure of the protein core and its relationship to the "collagen fibril binding site". In addition, the results here do not support the existence of the so-called "vertical components" of Scott's proposed orthogonal array of protein cores on the surface of the fibril. The need to interpret all stained glycosaminoglycan side chains as having collapsed against the protein core led Scott (1980) to propose that those oriented parallel to the long axis of the collagen fibril, across a D period, had collapsed against protein cores in extended conformation also lying axially (as vertical components) across all the bands of a D period. The existence of substantial numbers of these components would have led to a more random distribution of the ferritin particles than was observed here together with the probability that the protein core binds to the charged bands (like the *d* band) due to their similarity. The "vertical components" may be dye-bound glycosaminoglycans that have not collapsed against the protein core but against one another (or some other component) during dehydration. The distinct possibility that the protein core of PDS may be globular also contrasts with the extended conformation that Scott (1980) depicted in his scheme.

The proposed function of the protein core of PDS implies that proteoglycans possessing the same protein core but bearing L-iduronic acid-poor dermatan sulfate side chains might also be expected to bind to type I collagen fibrils. Whether proteoglycans with this protein core can also bind to types II and III fibrillar collagen is unknown but Vogel et al. (1984) demonstrated that the PDS from bovine flexor tendon does retard fibrillogenesis of type II collagen fibrils *in vitro*. It is also significant that a rabbit antiserum against the small proteoglycan extracted from fetal bovine epiphyseal cartilage localized the same, or a similar, antigen to the surface of type II collagen fibrils which may represent the component Orford and Gardner (1984) located at the *d* band of type II collagen fibrils with cuprolinic acid dye.

The proposed arrangement of PDS molecules on the surface of tendon collagen fibrils also implies that the nature of the surrounding polyanionic microenvironment is governed by the physical characteristics of the L-iduronic acid-rich dermatan sulfate side chains. Although they may possess subtle differences in copolymeric structure (Fransson et al., 1970), the most obvious difference in the L-iduronic acid-rich dermatan sulfate side chains of PDS molecules from different tissues is their length. Their size may influence the degree of hydration and the packing of fibrils into fibers. The relatively short dermatan sulfate side chains in mature bovine dermis may permit fibrils to pack closer together giving rise to its large fibers and fiber bundles.

In addition to varying their length, changing the content of L-iduronic acid residues in the dermatan sulfate side chains may provide the cell with an even greater ability to fine tune the properties of the polyanionic "cloud" covering the surface of the collagen fibrils. Although both L-iduronic acid-rich and poor dermatan sulfate can self-associate extracellularly (Fransson et al., 1979) the interactions between the L-iduronic acid-rich forms tend to be weaker (Fransson et al., 1982) and they also tend to bind more strongly to collagen (Obrink, 1972; Gelman and Blackwell, 1974). While L-iduronic acid-rich dermatan sulfate side chains, of any size, may promote fibril aggregation, L-iduronic acid-poor types may form a hydrated glycosaminoglycan aggregate covering the surface of the fibril and prevent fibril aggregation and the formation of large fibers. It is of interest that small proteoglycans bearing L-iduronic acid-poor dermatan

sulfate side chains were obtained from tissues containing small collagen fibers: human fetal membranes (Brennan et al., 1984), cartilage (Rosenberg et al., 1982) and arterial walls (Chang et al., 1983). It is also possible that the proteoglycans present in fetal membranes and arterial walls are associated with type III collagen fibrils. Rosenberg et al. (1982), based on immunoelectron microscopic studies proposed that the small proteoglycan from epiphyseal cartilage was not only on the surface of type II collagen fibrils but was present within the interfibrillar matrix as aggregates formed by the self-association of its dermatan sulfate side chains. This is difficult to reconcile with the proposed scheme that the presence of this particular protein core causes the proteoglycan to bind specifically to the collagen fibrils. As pointed out earlier, the rabbit antiserum used in these studies may not be specific for the same protein core recognized by the MAbs or, if produced against a proteoglycan with the same protein core, it may be crossreacting with a structurally similar but different molecule. Moreover, the model advanced by Rosenberg et al. (1982) to explain the formation of the aggregates would appear to depend on the presence of more than one glycosaminoglycan side chain.

Variation in the content of L-iduronic acid residues may also influence the movement of cells over the surface of the fibrils. Cell attachment and spreading has been postulated to involve the destabilization of heparan sulfate rich adhesion sites by chondroitin sulfate and hyaluronic acid (Laterra et al., 1979). Cultured fibroblasts retain a chondroitin sulfate proteoglycan in the cell layer during periods of exponential growth which gives way to a dermatan sulfate-containing proteoglycan once they reach confluency and enter a quiescent G_0 phase (Dietrich et al., 1982). Transformed cells do not make this switch (Dietrich et al., 1982) which might be related to their increased motility. Using affinity chromatography, cell surface dermatan sulfate was the only galactosaminoglycan capable of displacing heparan sulfate from fibronectin (Stamatoglou and Keller, 1982). This may be interpreted to suggest that it is also involved in cell adhesion rather than increased motility. Whether the degree of adhesion by cells to the collagenous matrix may be subtly influenced by the content of L-iduronic acid in the

polyanionic "cloud" above the collagen fibrils remains to be determined.

4.6 Suggestions for Further Study

The 4 monoclonal antibodies that recognize the protein core of bovine skin PDS should continue to be highly useful in further studies of its structure. An obvious use of the MAbs would be to aid in the amino acid sequencing of the protein core. Their recognition of distinct epitopes may serve to identify those fragments of the protein core (on immunoblots) that share a common sequence. Based on the recognition of overlapping fragments by several of the MAbs and their size, their proximity to one another may be determined. Such an analysis has already indicated the probable order of the major CNBr peptides within the protein core. The MAbs could also be used to make affinity columns to isolate a particular fragment or several fragments of varying size that obviously overlap by virtue of the epitope. Analysis of such a group of fragments would establish the sequence in the area of the epitope and blocks of sequence could be determined based on the number of epitopes available. Use of 5D1, known to recognize a fifth epitope, would make this approach even more useful. And, as a result of the sequencing of the protein core, it will be possible to locate and characterize the epitope of each MAb more precisely.

The structure of the various PDS proteoglycans isolated to date should be characterized further, both chemically and immunologically. The average molecular weight of the dermatan sulfate side chains should be determined and the molecule analyzed on immunoblots after digestion with cathepsin C and CNBr to verify that they all possess a structurally similar protein core with a single dermatan sulfate side chain at their NH_2-terminus.

The MAbs would prove useful in immunohistochemically studying the biosynthesis of the protein core of PDS in dermal or gingival fibroblasts. Its biosynthetic pathway through the cell to its secretory granules could be followed, as well as its release and uptake by collagen fibrils if the cells were cultured on a collagen gel. Used together as an antiserum the MAbs may be able to immunoprecipitate a putative biosynthetic precursor of the protein core of PDS which

then may be characterized further.

The MAbs could be used to evaluate the structural relationship between the protein core of PDS and those of other apparently different proteoglycans such as proteokeratan sulfate, proteoheparan sulfate and small proteochondroitin sulfates. Of particular interest is the small proteochondroitin sulfate isolated from bovine nasal cartilage by Heinegård et al. (1981). Its amino acid composition is very similar to that of PDS, but it has been proposed that this molecules contains at least two rather long chondroitin sulfate side chains. However, priority should be given to the study of those proteoglycans containing L-iduronic acid poor dermatan sulfate side chains that appear to contain the same protein core as PDS in order to test the notion that this protein core has only a single galactosaminoglycan side chain attached. Both the bovine fetal epiphyseal cartilage proteoglycan (Rosenberg et al. 1982) and the proteoglycan synthesized by bovine arterial smooth muscle cells could be analyzed by the MAbs on immunoblots after digestion with CNBr, cathepsin C and chondroitinases AC II and ABC. In addition, the MAbs could be used to immunohistochemically locate the protein core in adult bovine articular cartilage to confirm the results of Orford and Gardner (1984) and to determine whether or not this protein core is located in the interfibrillar spaces. The ability of the MAbs to define a protein core with a particular function (i.e., bind to collagen fibrils) would be important in helping to classify proteoglycans and would represent a large step in unifying much of the data regarding these small proteoglycans containing dermatan sulfate.

It may be possible to estimate semi-quantitatively the amount of (ABC)core present on an immunoblot if, instead of applying the diaminobenzidine, the portion of the nitrocellulose containing the (ABC)cores was cut out and placed in a flat-bottomed polystyrene well with o-phenylenediamine. The amount of antigen present could then be related to a series of standards as measured by absorbance at 492 nm. If this method proved effective it seems possible that the functional and biosynthetic relationship between the proteoglycans varying in L-iduronic acid content could also be studied by examining the culture fluid of fibroblasts. For example, one may be able to relate the amount of the protein core synthesized by a certain type

of cell to the amount and type of collagen it also synthesizes. This would serve as a means to determine whether synthesis of the protein core is associated with all three major interstitial collagens. Immunoprecipitation of the intact proteoglycan and its treatment with chondroitinase AC II and subsequent immunblotting would reveal the nature of its glycosaminoglycan side chain. Changes in the type of collagen synthesized may then be related to any concommitant shift in the L-iduronic acid content of its galactosaminoglycan side chains. Similarly, as chondroblasts dedifferentiate to fibroblasts and synthesize type I collagen, the nature of a potential change in the glycosaminoglycans may be detectable. Such changes in the nature of the glycosaminoglycan may also be detectable during healing skin wounds.

The MAbs have indicated that the protein core of PDS binds preferentially to collagen fibrils in the vicinity of the *d* and *e* bands, very likely between them, and it has been suggested that the protein core binds only to fibrillar collagen. Studies are in progress (Pearson and Scott, unpublished results) that are designed to test this notion and the MAbs have been useful in developing methods to remove as much PDS from the collagen sample as possible. If PDS can bind to preformed collagen fibrils in a normal manner (as verified immunohistochemically) then it may be possible more accurately to locate the binding site on collagen fibrils by affinity labeling methods using chemically modified PDS or perhaps the large CNBr fragment, should it also bind.

Such preformed fibrils could also be used to evaluate the binding efficiency of proteoglycans varying in L-iduronic acid content. This would reveal whether L-iduronic acid poor-dermatan sulfate side chains play a role in modulating the binding of the protein core to collagen fibrils; possibly reducing the binding as suggested by the results of Rosenberg et al. (1982). This study could be expanded to include types II and III collagens as the L-iduronic acid content may play a role in binding specificity. Moreover, fibrils coated with these different forms of the proteoglycan could be used as a substrate upon which to culture fibroblasts to determine whether they affect cell behavior in any way. Of interest would be the effect the L-iduronic acid content on the rate of cell attachment and spreading.

If it could be demonstrated that collagen fibrils formed *in vitro* are suitable substrates for calcification then it might be possible to determine to what extent the presence of PDS in the gap zone inhibits collagen mineralization. It would be interest to determine whether the protein core itself has bound to a critical location in the gap zone or whether the nature of the polyanionic galactosaminoglycan side chains plays a dominant role in this process. The observed lack of dye-stained components associated with the *d* band of demineralized collagen fibrils (Scott, 1984) suggests that PDS and related proteoglycans bound to collagen in the gap zone may prevent soft tissue calcification.

As the gaps between collagen molecules in the gap zone are delimited by the N- and C-terminal telopeptides which are involved in forming intermolecular crosslinks, PDS bound to collagen fibrils in this region may influence their conformation (Helseth and Veis, 1979) and the extent to which they participate in crosslinking. PDS molecules lying in the gap zone may play a very important role in collagen turnover as it may be necessary to remove the PDS prior to enzymic degradation of the collagen. It would be of great interest to study the effect bound PDS has on the efficacy of purified enzyme preparations that are responsible for the scission of the telopeptides from the collagen monomers.

Lastly, PDS and related proteoglycans could be used to repeat the study of Vogel et al. (1984) to determine how the L-iduronic acid content of the galactosaminoglycans, attached to the same protein core influence fibrillogenesis *in vitro*. And, as PDS binds to the fibrils within the gap zone, it would be of great interest to use pC and pN collagen molecules in these experiments as the presence of the propeptides would seem to preclude the possibility of PDS binding to these fibrils.

Plate 1. Immunoblot of mature bovine skin PDS and its (HF)core. Intact PDS (lanes 2, 4, 6, 8, 10 and 13) and PDS that had been treated with anhydrous HF (lanes 3, 5, 7, 9, 11 and 14) were transferred from a 5% SDS-polyacrylamide slab gel to nitrocellulose paper and stained with Amido Black (lanes 2 and 3) or immunochemically (lanes 4-14) using 1:1000 dilutions of monoclonal antibody ascites fluids (as indicated) or a 1:100 dilution of normal mouse serum (NMS). 15 μg (protein) was applied to lanes 2 and 3, 1 μg to lanes 4, 5, 13 and 14, 500 ng to lanes 6-9 and 300 ng to lanes 10 and 11. 200 ng of the (ABC)core of PDS was applied to lane 12.

Plate 2. Immunoblot of intact bovine skin PDS (lanes 3 and 9) and its (ABC)core (lanes 2, 4-8) using 1:1000 dilutions of MAb ascites fluids or a 1:100 dilution of normal mouse serum as indicated. Samples were transferred from a 7% gel. 10 μg (protein) (ABC)core was applied to lane 2 and 1.5 μg to lanes 4-8. The (ABC)core was prepared by N. Winterbottom according to the method of Oike et al. (1980) using 0.33 units enzyme/mg sample, dialyzed free of GdnHCl and small digestion products by ultrafiltration and lyophilized.

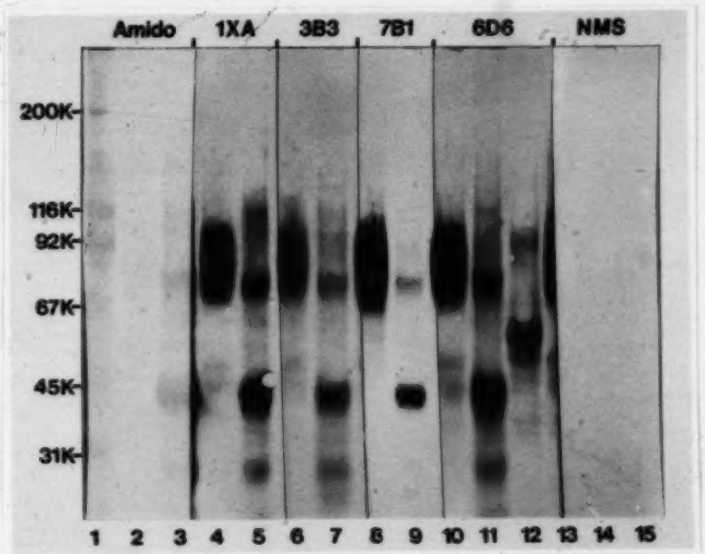

Plate 1.

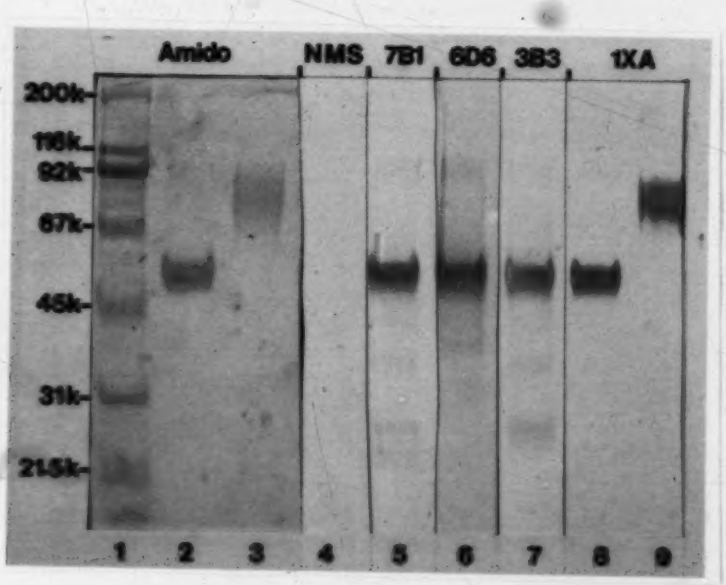

Plate 2.

Plate 3. Immunoblot of bovine skin PDS (lane 7, 300 ng protein) and PDS that had been treated with a β-elimination, sulfite addition reaction (lanes 2-6 and 9). Lanes were stained immunochemically or with Amido black as indicated. Samples were transferred from a 5% SDS-polyacrylamide slab gel. 20 μg protein was applied to lane 2, 1 μg to lanes 3 and 9, 500 ng to lanes 4 and 5 and 300 ng to lane 6. 200 ng (protein) of the (ABC)core preparation of PDS was applied to lane 8.

Plate 4. Immunoblot of concentrated (about 50-fold) culture medium from confluent human gingival fibroblasts (HUGE 1, passage 7) that had been incubated with 0.05 μg/ml tunicamycin. 10 μl was applied to lanes 2 and 3. 10 μl of medium that had been incubated with 0.05 units of chondroitinase ABC/ml medium for 1 hr at 37°C was applied to lane 4. Bovine skin PDS (200 ng; lane 5) and its (ABC)core (60 ng; lane 6) were also blotted. Samples were transferred from a 7% gel. 1XA and 3B3 also stained a component (M_r=43,000) in the enzyme treated medium, but extremely faintly. The blots treated with 7B1 and NMS were negative.

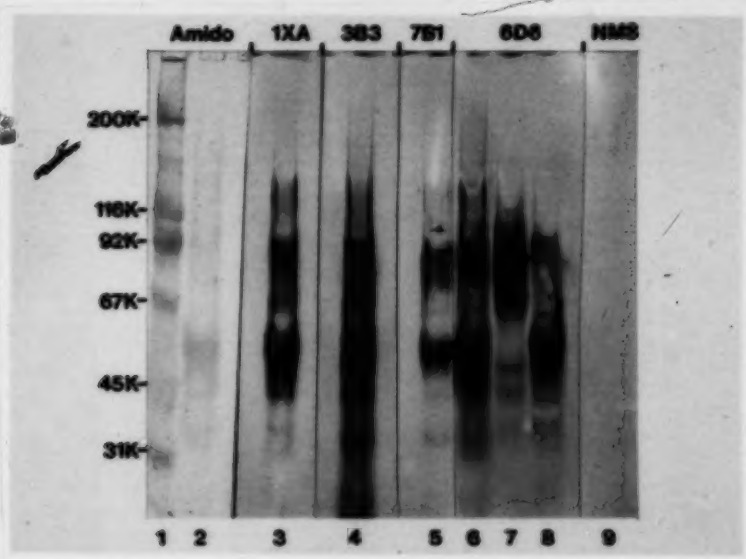

Plate 3.

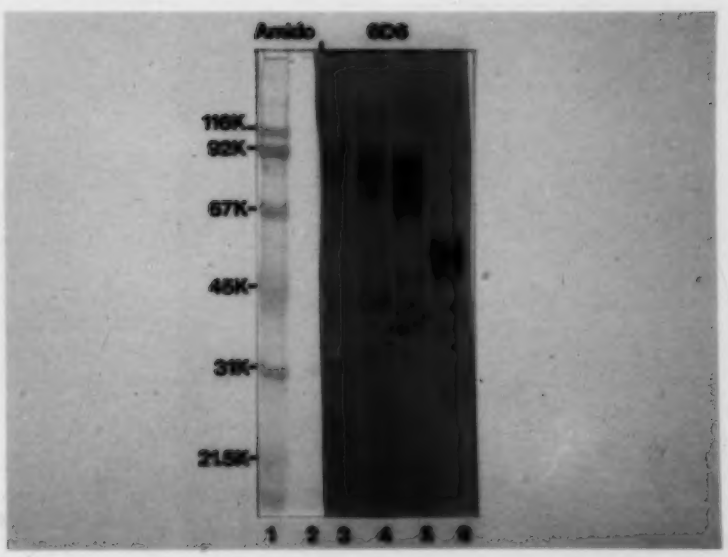

Plate 4.

Plate 5a. Immunoblot of bovine skin PDS treated with cathepsin C. Aliquots of the digest were removed immediately (0 min) (lanes 5, 7, 9 and 11) and after 6 min (lanes 4, 6, 8, 10 and 12) and 30 min of digestion (lane 13), diluted in sample buffer and boiled for 10 min. 3.4 μg protein was applied to lane 4; 100 ng to lanes 5-13. Intact PDS (lanes 2 and 14) and its (ABC)core preparation (lane 15) were also transferred from the 7% gel. The lanes were stained immunochemically as indicated.

Plate 5b. Immunoblot of bovine skin PDS treated with cathepsin C and stained with 7B1. Aliquots of the digest were removed after 6 min (lanes 4 and 5) and 30 min (lanes 2 and 3) of digestion, diluted in sample buffer and boiled. 100 ng protein was applied to lanes 2-5. An (ABC)core preparation of PDS (lane 1, 100 ng) was also transferred from the 10% gel. The lanes were stained immunochemically with a 1:1000 dilution of 7B1 ascites fluid.

Plate 6. Immunoblot of bovine skin PDS that had been treated with trypsin for 2 hr at 37°C (lanes 5, 7, 9, 11 and 15; 250 ng protein/lane) or cathepsin D for 1 hr at 37°C (lanes 4, 6, 8, 10 and 14; 240 ng protein/lane) and stained immunochemically as indicated. About 5.5 μg protein of the cathepsin D-digested PDS was applied to lane 3. Intact PDS (100 ng, lane 12) and its (ABC)core preparation (60 ng, lane 13) were also transferred from the 10% gel.

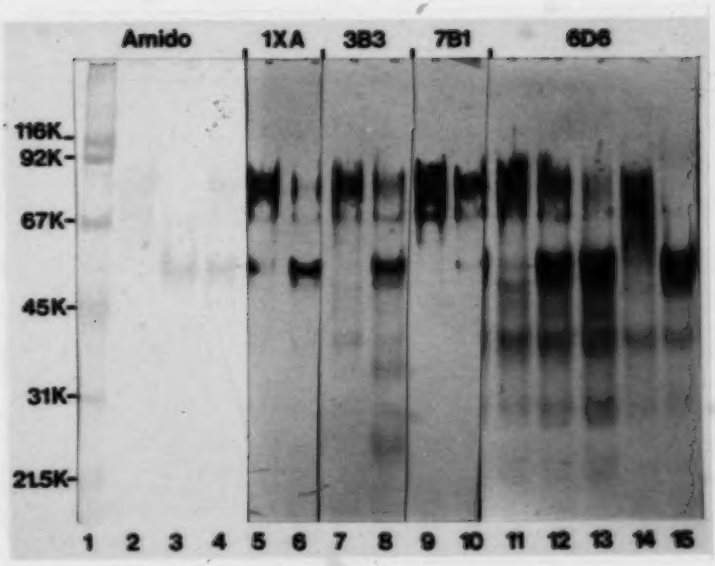

Plate 5a.

Plate 5b.

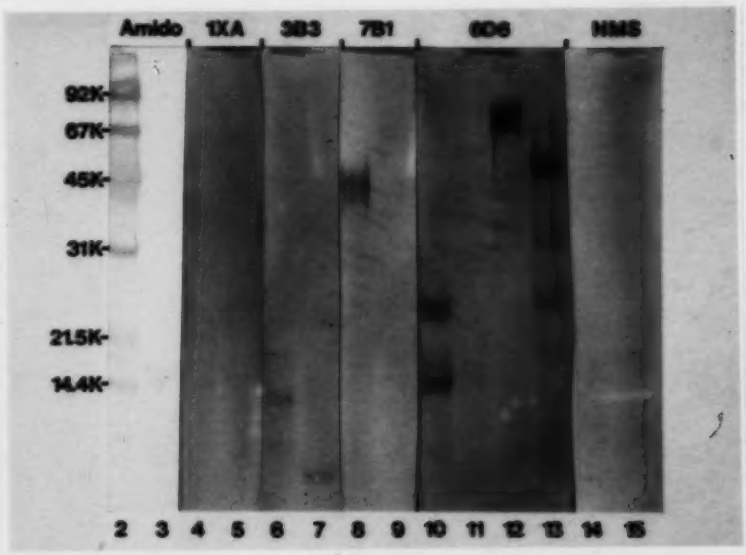

Plate 6.

Plate 7. Immunoblot of bovine skin PDS fragments. Cathepsin D-digested PDS was applied to lanes 2 (10 μg protein), 4 (2 μg), 5, 6 and 11 (1 μg) and 14 (6 μg) with (lanes 4, 5 and 11) and without (lane 6) prior reduction in sample buffer. 2 μg of cathepsin D-digested PDS that had been subsequently digested with chondroitinase ABC (0.005 units) was applied to lane 12. The insoluble fraction of a cyanogen bromide digest of PDS was applied to lanes 3 (5 μg), 7-9 (1 μg) and 13 (5 μg). Samples in lanes 3, 7, 9 and 13 were reduced with 2-mercaptoethanol. 2 μg of the cyanogen bromide digest that had been digested with chondroitinase ABC (0.005 units) was applied to lane 10. The samples were transferred from a 10% gel and stained immunochemically or chemically with Amido black or periodic acid-Schiff stain (PAS) as indicated.

Plate 8. Immunoblot of the cyanogen bromide cleavage products of bovine skin PDS. The cyanogen bromide digest (supernatant fraction) was applied to lanes 3-8 and intact PDS was applied to lanes 2 (10 μg) and 9 (2 μg). The samples were transferred from a 10% gel. 7 μg protein was applied to lane 3, 13 μg to lane 4 and 2 μg to lanes 5-8. The lanes were stained immunochemically with MAb ascites fluids or normal mouse serum as indicated.

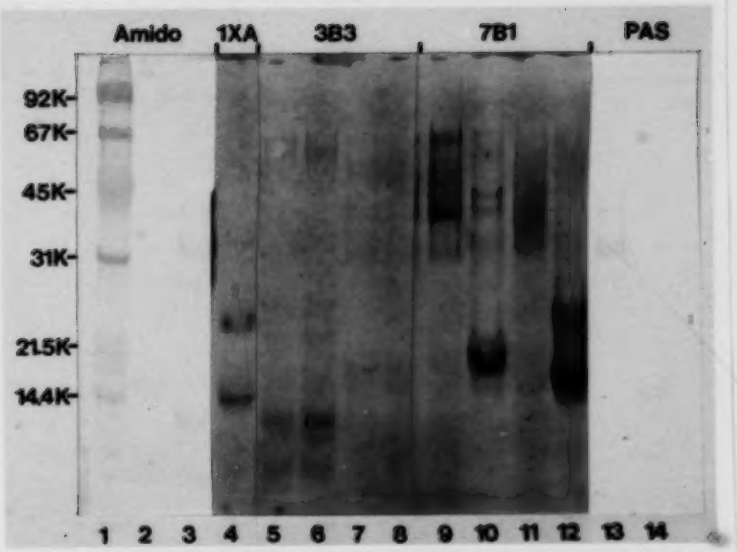

Plate 7.

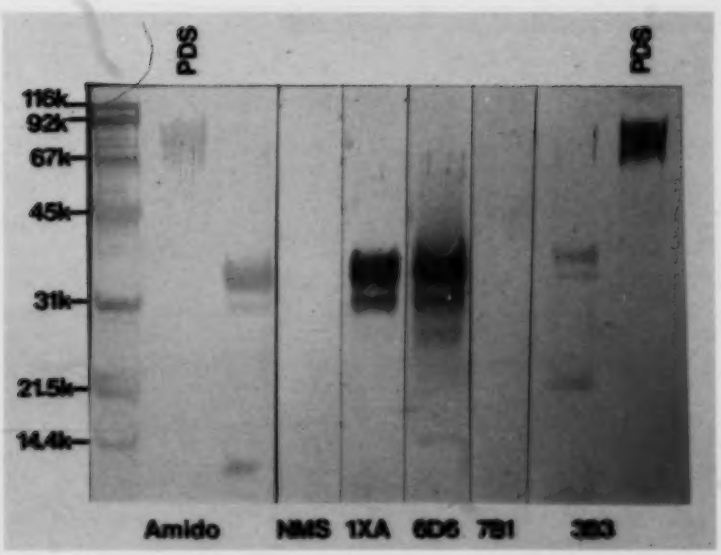

Plate 8.

Plate 9. Immunoblot of the cyanogen bromide peptides of PDS previously treated with anhydrous HF. This material was prepared and generously provided by N. Winterbottom. 16 μg (protein) of the cyanogen bromide digest was applied to lane 3 and 4 μg applied to lanes 4-8 and transferred from a 10% gel. 6 μg (protein) of intact PDS was applied to lanes 2 and 9. The lanes were stained chemically or immunochemically with the MAb ascites fluids (1:1000) or normal mouse serum (1:100) as indicated.

Plate 10. Nitrocellulose electrophoresis of glycosaminoglycan-bearing cyanogen bromide peptide(s) isolated by ion exchange and gel filtration chromatography. The sample was dissolved in a 0.06M sodium barbital, pH 8.6 buffer and 10 μg was applied to a 2.5 x 10 cm strip of nitrocellulose paper (moistened with buffer) in 4 μl (strip 2). 15 μg of the dermatan sulfate standard was applied to strip 1. Electrophoresis proceeded at room temperature for 1 hr using 0.8 mA/strip. The dermatan sulfate standard was stained with toluidine blue while strip 2 was treated with CPC for 30 min, washed in water and stained immunochemically with 7B1.

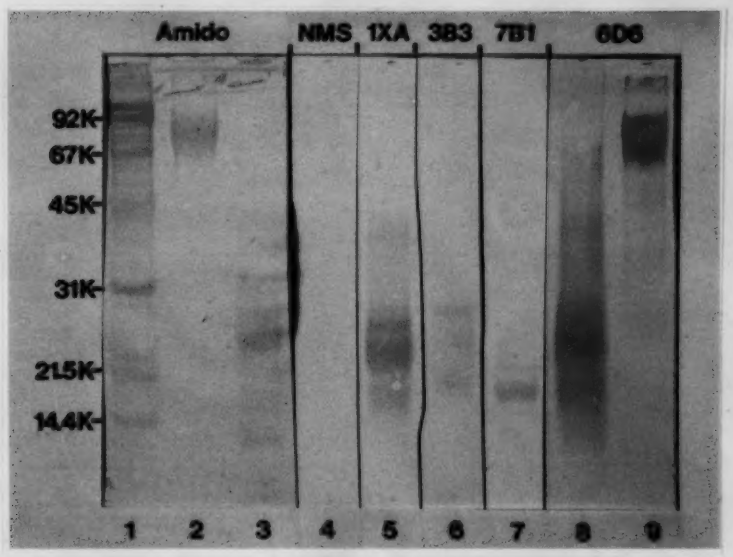

Plate 9.

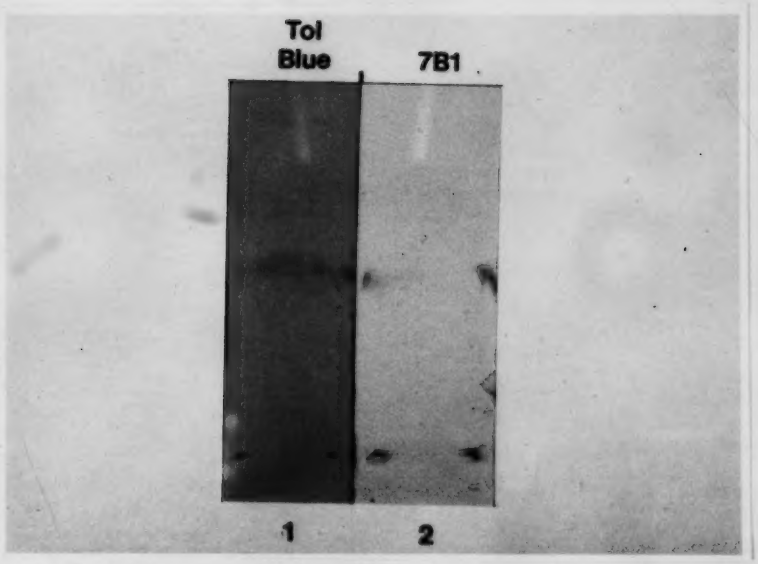

Plate 10.

Plate 11. Nitrocellulose electrophoresis of the insoluble fraction of a cyanogen bromide digest of bovine skin PDS (previously shown to contain the same components as the soluble fraction). 150 μg of the digest was dissolved in 15 μl of a 0.06M sodium barbital, pH 8.6 buffer. Samples were applied in 4 μl aliquots to 2.5 x 10 cm strips of nitrocellulose paper moistened with buffer. 15 μg of the dermatan sulfate standard was applied to strip 1. 40 μg of the digest was applied to strips 2 and 3 and 10 μg of the digest was applied to strips 4-8. Electrophoresis proceeded for 1 hr at room temperature using 0.8 mA/strip. Strips 1-3 were stained with dye as indicated while strips 4-8 were treated with 0.1% CPC for 30 min, washed in water and stained immunochemically with the MAbs as indicated.

Plate 12. Immunoblot of bovine periodontal ligament PDS. 19.5 μg (protein) of the sample was dissolved in 39 μl of 0.1M sodium acetate, 0.1M Tris HCl, pH 7.4 buffer. Chondroitinase ABC (0.1 units/mg sample) was added to half of the sample and both portions were incubated for 2 hr at 37°C. Appropriate amounts of both samples were diluted with SDS-PAGE sample buffer (containing 10% 2-mercaptoethanol), boiled for 5 min and applied to a 5% slab gel. Undigested sample was applied to lanes 2 (4 μg), 4, 6 and 14 (200 ng) and lanes 8 and 10 (100 ng). The enzyme treated sample was applied to lanes 3 (4 μg), lanes 5, 7 and 15 (200 ng) and lanes 9 and 11 (100 ng). 80 ng (protein) of bovine skin PDS and its (ABC)core preparation were applied to lanes 12 and 13, respectively. The lanes were stained with Amido black or immunochemically with the MAb ascites fluids or normal mouse serum as indicated.

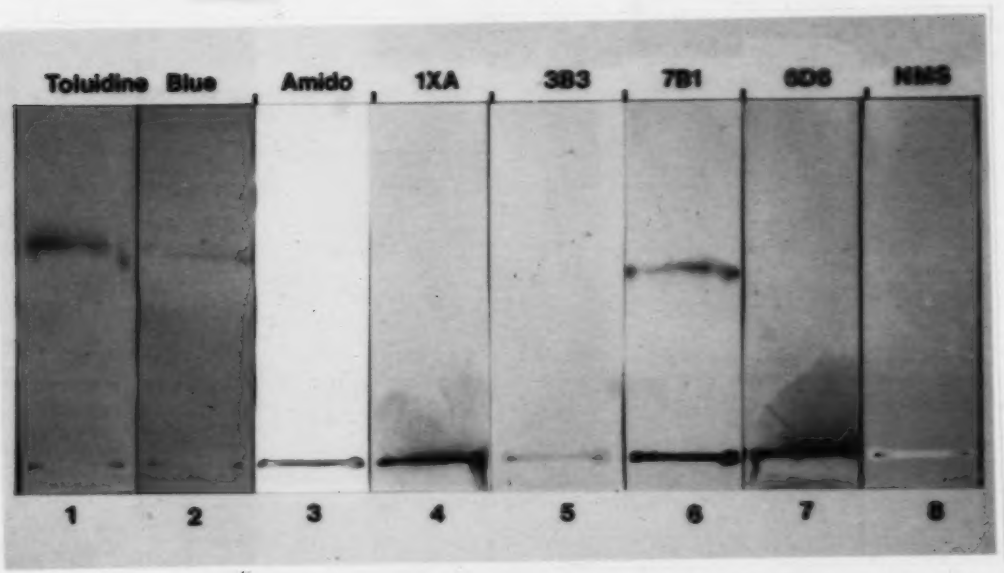

Plate 11.

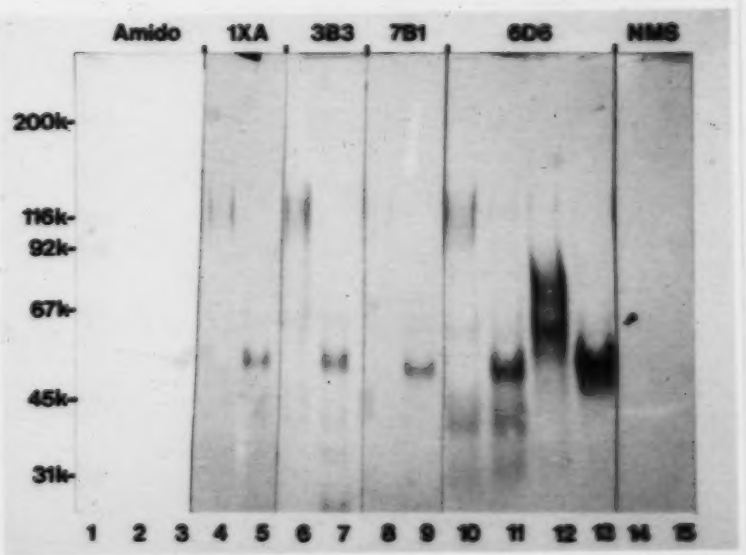

Plate 12.

Plate 13. Immunoblot of bovine gingiva PG2. The sample was dissolved in 0.1M sodium acetate, 0.1M Tris HCl, pH 7.4 buffer at a concentration of 1 μg protein/μl. Chondroitinase ABC (0.1 units/mg sample) was added to one half of the sample and both portions were incubated for 55 min at 37°C. Appropriate amounts of each sample were diluted with SDS-PAGE sample buffer (containing 10% 2-mercaptoethanol) and boiled for 5 min. 15 μg (protein) of the undigested sample was applied to lane 2, 1 μg to lanes 4 and 14, 500 ng to lanes 6 and 8 and 300 ng to lane 10. 12 μg of the enzyme treated sample was applied to lane 3, 1 μg to lanes 5 and 15, 500 ng to lanes 7 and 9 and 300 ng to lane 11. 300 ng of bovine skin PDS and 100 ng of its (ABC)core preparation were applied to lanes 12 and 13, respectively. The samples were transferred from a 5% slab gel and stained with Amido black or immunochemically as indicated.

Plate 14. Immunoblot of bovine fetal skin PDS. The sample was dissolved in buffer at a concentration of 100 ng protein/μl. Chondroitinase ABC (0.1 units/mg sample) was added to one half of the sample and both portions were incubated for 1 hr at 37°C. Appropriate amounts of each sample wer diluted with SDS-PAGE sample buffer (containing 10% 2-mercaptoethanol) and boiled for 5 min. The undigested sample was applied to lanes 2 (3 μg protein) and lanes 4, 6, 8, 10 and 14 (100 ng). The enzyme treated sample was applied to lane 3 (3 μg) and lanes 5, 7, 9, 11 and 15 (100 ng). 50 ng of adult bovine skin PDS and its (ABC)core preparation were applied to lanes 12 and 13, respectively. The samples were transferred from a 7% gel and stained as indicated.

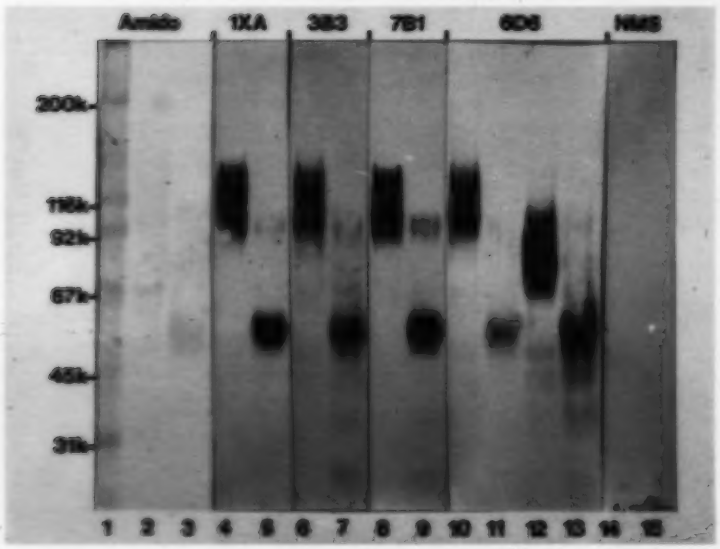

Plate 13.

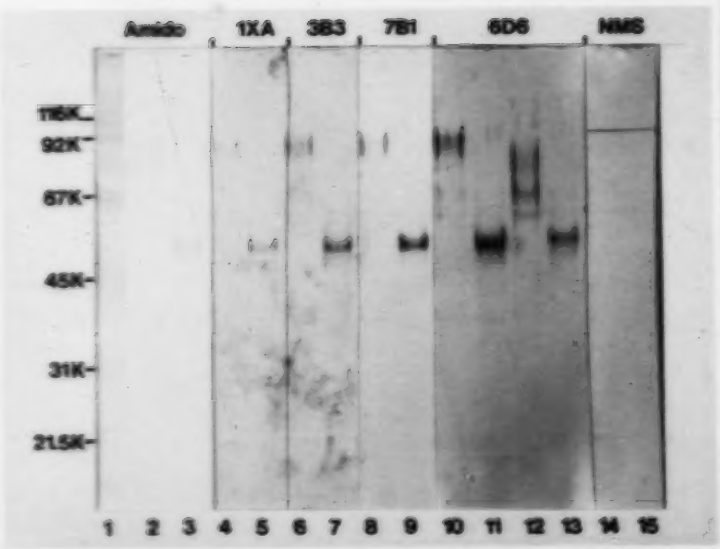

Plate 14.

Plate 15. Immunoblot of human gingiva PG2. The sample (sample 31, from 'most diseased human gingiva') was dissolved in 0.1M sodium acetate, 0.1M Tris HCl, pH 7.4 buffer at 500 ng protein/μl and chondroitinase ABC (0.1 units/mg sample) was added to one half of the sample and both portions incubated at 37°C for 50 min. Appropriate amounts of each sample were diluted with SDS-PAGE sample buffer(containing 10% 2-mercaptoethanol) and boiled for 5 min. The undigested sample was applied to lanes 2 (10 μg), 4, 6, 8, 10 and 14 (1 μg) while the enzyme treated sample was applied to lanes 3 (10 μg), 5, 7, 9, 11 and 15 (1 μg) of the 5% gel. The transferred samples were stained with Amido black or immunochemically as indicated.

Plate 16. Immunoblot of adult human skin PDS and its protein core. Human skin PDS was dissolved and boiled in SDS-PAGE sample buffer (containing 10% 2-mercaptoethanol) at a concentration of 100 ng protein/μl. 4 μg was applied to lane 2, while 200 ng was applied to lanes 4, 6, 8, 10 and 14. Human skin PDS protein core, produced by treatment with alkali, was dissolved (by boiling) in SDS-PAGE sample buffer at the same concentration. 4 μg (protein) was applied to lane 3, while 200 ng was applied to lanes 5, 7, 9, 11 and 15. 100 ng of bovine skin PDS and an (ABC)core preparation of PDS were applied to lanes 12 and 13, respectively. The samples were transferred to nitrocellulose paper from a 10% slab gel and stained with Amido black or immunochemically as indicated.

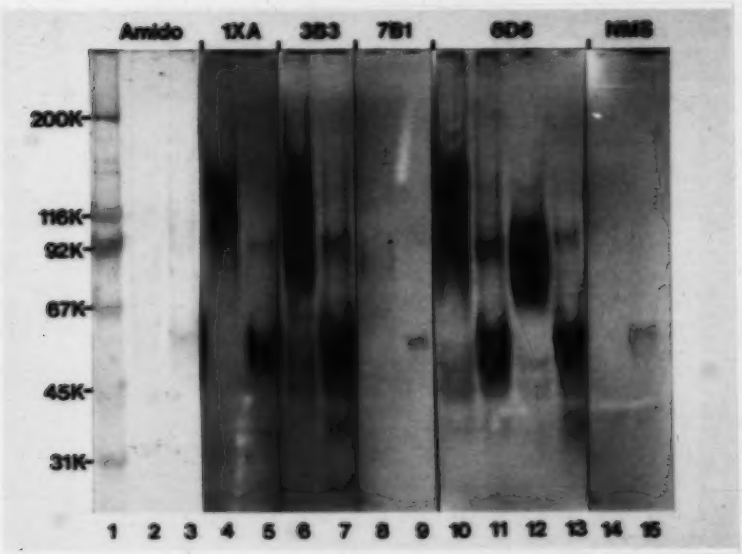

Plate 15.

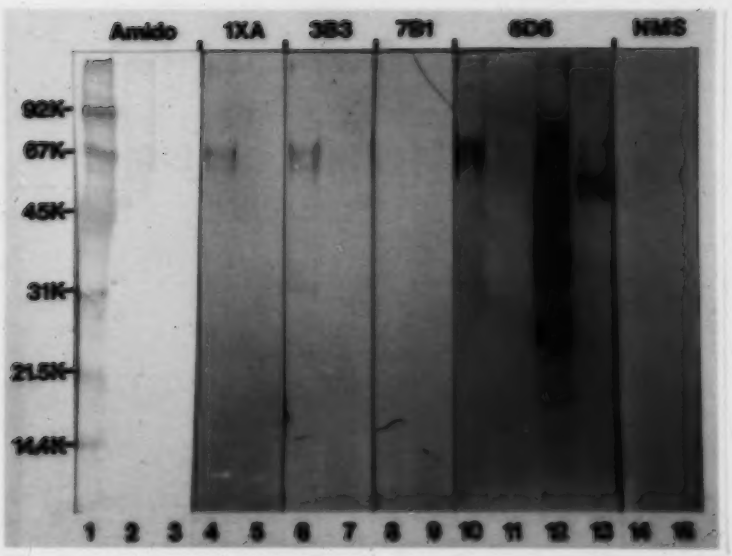

Plate 16.

Plate 17. Immunoblot of concentrated (about 200-fold) culture medium from confluent Gin-1 human gingival fibroblasts. 30 μl of medium was diluted with an equivalent volume of SDS-PAGE sample buffer (containing 10% 2-mercaptoethanol), and after boiling for 5 min, was applied to lanes 6, 8, 10 and 12. 30 μl of medium, previously digested with chondroitinase ABC (see methods section), was diluted in the sample buffer, boiled and applied to lanes 7, 9, 11 and 13. Bovine skin PDS was applied to lanes 2 (7 μg), 5 (2.5 μg) and 14 (1 μg). An (ABC)core preparation of PDS was applied to lanes 3 (10 μg) and 15 (1 μg). 10 μg of a crude sample of human gingival PDS was also applied to lane 4. The samples were transferred to nitrocellulose paper from a 7% gel and stained with Amido black or immunochemically with the MAb ascites fluids or normal mouse serum as indicated.

Plate 18. Immunoblot of concentrated medium (about 15-fold) from confluent monkey arterial smooth muscle cells. 60 μl of medium in SDS-PAGE sample buffer was applied to lane "SMC", while 60 μl of medium, previously incubated with chondroitinase ABC for 1 hr at 37°C, was applied to the next lane labelled "Core". Bovine skin PDS (2.5 μg) and its (ABC)core preparation (2 μg) were also applied to the 5% gel as indicated. The transferred samples were stained immunochemically with 6D6 ascites fluid as usual.

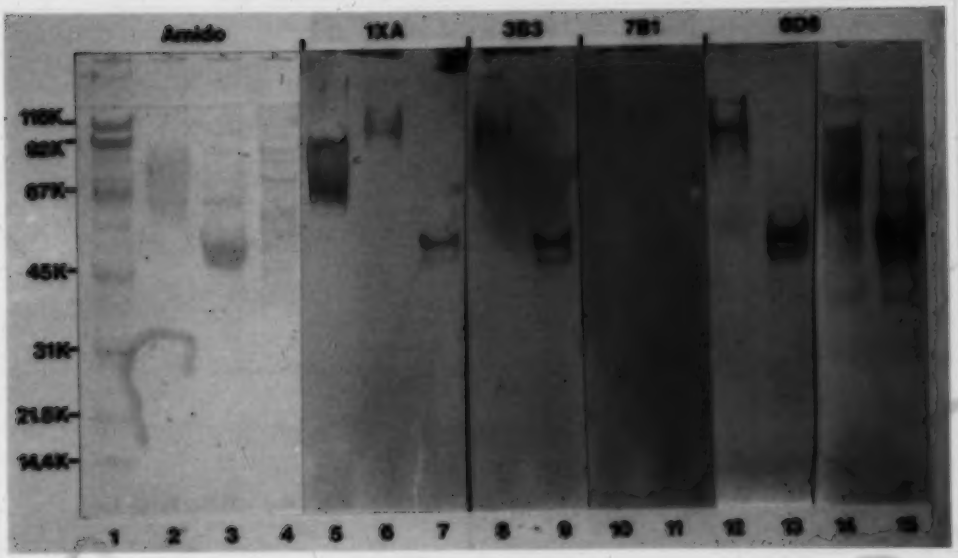

Plate 17.

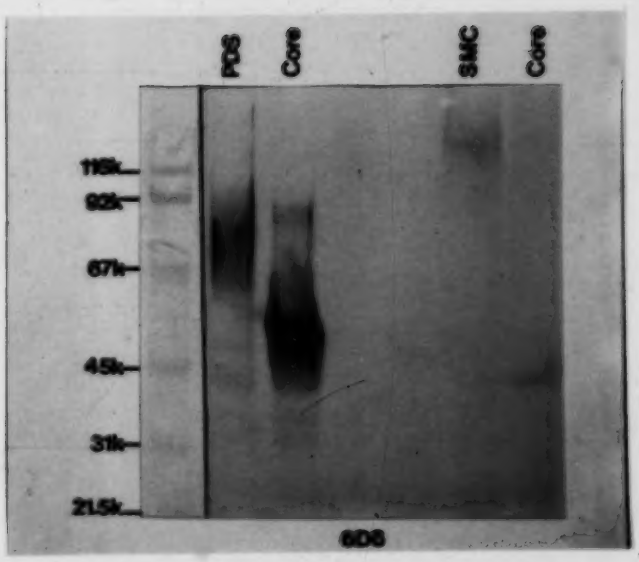

Plate 18.

Plate 19. Immunoblot of the A1D1 fraction from the Swarm rat chondrosarcoma. The sample, previously dissolved in 0.1M sodium acetate, 0.1M Tris HCl, pH 7.4 buffer, was diluted in SDS-PAGE sample buffer (containing 5% 2-mercaptoethanol) and, after boiling for 5 min, was applied in 14 μg aliquots to lanes marked "U". 14 μg of sample, previously incubated with chondroitinase ABC (0.2 units/mg sample), were also applied to lanes marked "E". 500 ng of bovine skin PDS and 1 μg of its (ABC)core preparation were also applied. The samples were transferred to nitrocellulose paper from a 5% slab gel and stained with Amido black or immunochemically with the MAb ascites fluids (1:1000) or normal mouse serum (1:100) as indicated.

Plate 20. Immunoblot of fraction A1D1 isolated from bovine nasal cartilage. The A1D1 sample was diluted in SDS-PAGE sample buffer (without 2-mercaptoethanol), boiled for 4 min and 11 μg (protein) were applied to lanes labelled "U". 10 μg of A1D1, previously incubated with chondroitinase ABC (as described above), were boiled and applied to lanes labelled "E". 50 ng of bovine skin PDS and 30 ng of its (ABC)core preparation were applied to the lanes indicated. The samples were transferred from a 7% gel and stained with Amido black or immunochemically as indicated.

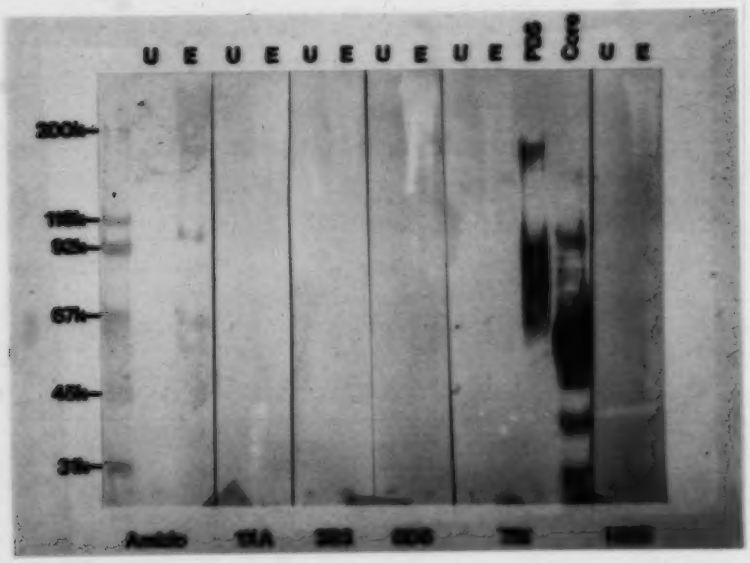

Plate 19.

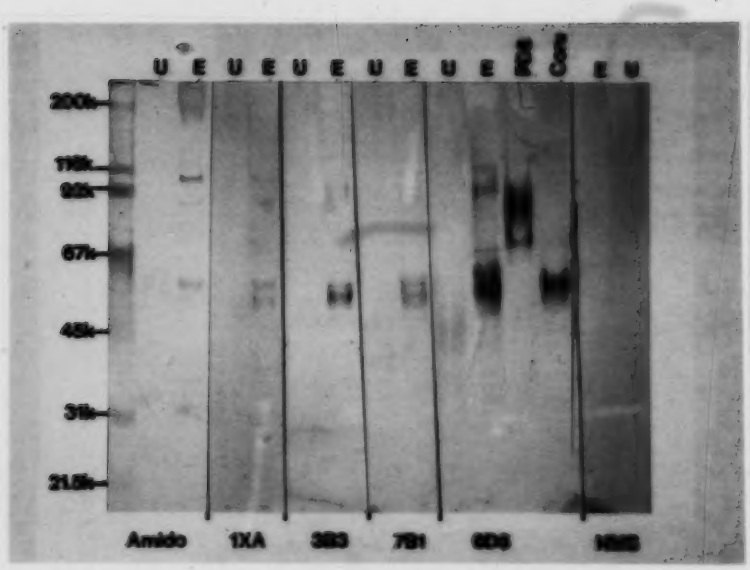

Plate 20.

Plate 21. Immunoblot of the A1D4 fraction extracted from the Swarm rat chondrosarcoma. 8 μg (protein) of A1D4, diluted in SDS-PAGE sample buffer containing 10% 2-mercaptoethanol and boiled for 3 min, was applied to lanes labelled "U". 10 μg (protein) of A1D4 that had been incubated with chondroitinase ABC (as described above) was similarly diluted and boiled and applied to lanes labeled "E". 1.25 μg of bovine skin PDS and 2 μg of its (ABC)core preparation were also applied to the lanes indicated. The samples were transferred from a 7% gel and stained as indicated.

Plate 22. Immunoblot of the A1D4 fraction extracted from bovine nasal cartilage. 15 μg (protein) of the sample, diluted in SDS-PAGE sample buffer (without 2-mercaptoethanol) and boiled for 5 min, were applied to lanes labelled "U", while 15 μg (protein) of A1D4 that had been incubated with chondroitinase ABC (0.2 units/mg sample) for 8 hr at 37°C was applied to lanes labeled "E". 100 ng of bovine skin PDS and 60 ng of its (ABC)core preparation were applied to the lanes indicated. Samples were transferred to nitrocellulose paper from a 7% slab gel and stained with Amido black or immunochemically as usual.

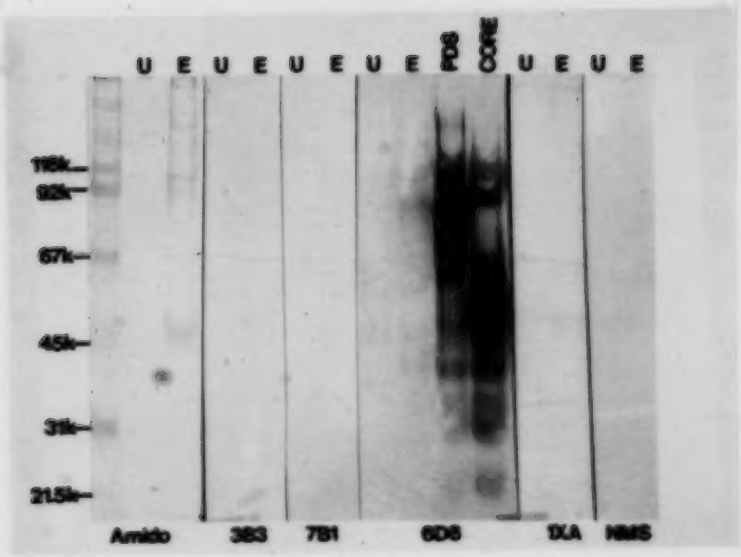

Plate 21.

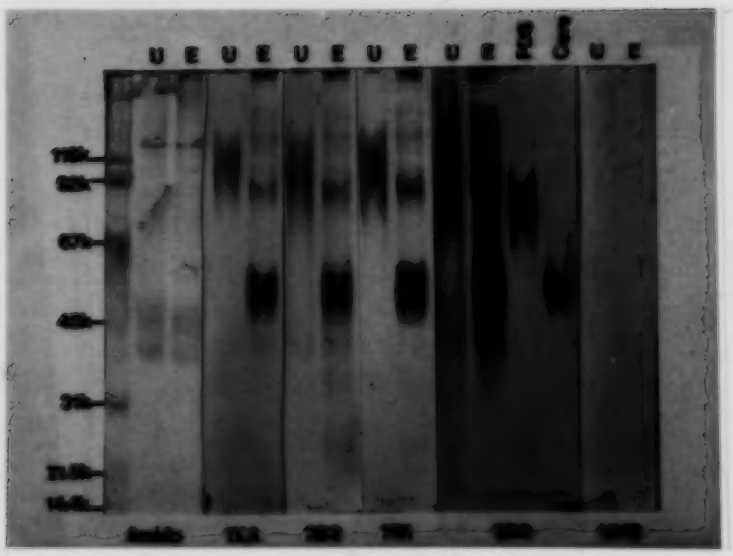

Plate 22.

Plate 23. Immunoblot of the A1D4 fraction extracted from chick sternal cartilage. 10 μg (protein) of the A1D4 fraction diluted in SDS-PAGE sample buffer containing 10% 2-mercaptoethanol and boiled were applied to lanes labelled "U". 10 μg of A1D4 that had been incubated with chondroitinase ABC for 8 hr at 37°C was applied to lanes labelled "E". 10 μg of the A1D4 fraction from bovine nasal cartilage (BNC) and its (ABC)core preparation were applied to the indicated lanes, as were 1.25 μg of bovine skin PDS and its (ABC)core preparation. The samples were transferred to nitrocellulose from a 7% gel and stained with Amido black or immunochemically as usual.

Plate 24. Immunoblot of bovine dental pulp PG2. The sample was dissolved in 0.1M sodium acetate, 0.1M Tris HCl, pH 7.4 buffer and chondroitinase ABC (0.1 unit/mg sample) was added to one half of the sample and then both portions were incubated for 50 min at 37°C. The reduced, undigested sample was applied to lanes 2 (16 μg), 4 and 14 (5 μg), 6 and 8 (3 μg) and 10 (1 μg). The reduced enzyme treated sample was applied to lanes 3 (12 μg), 5 and 15 (5 μg), 7 and 9 (3 μg) and 11 (1 μg). 300 ng of bovine skin PDS and 100 ng of its (ABC)core preparation were applied to lanes 12 and 13, respectively. The samples were transferred from a 5% gel and stained with Amido black or immunochemically with MAb ascites fluids (1:1000) or normal mouse serum (1:100) as indicated.

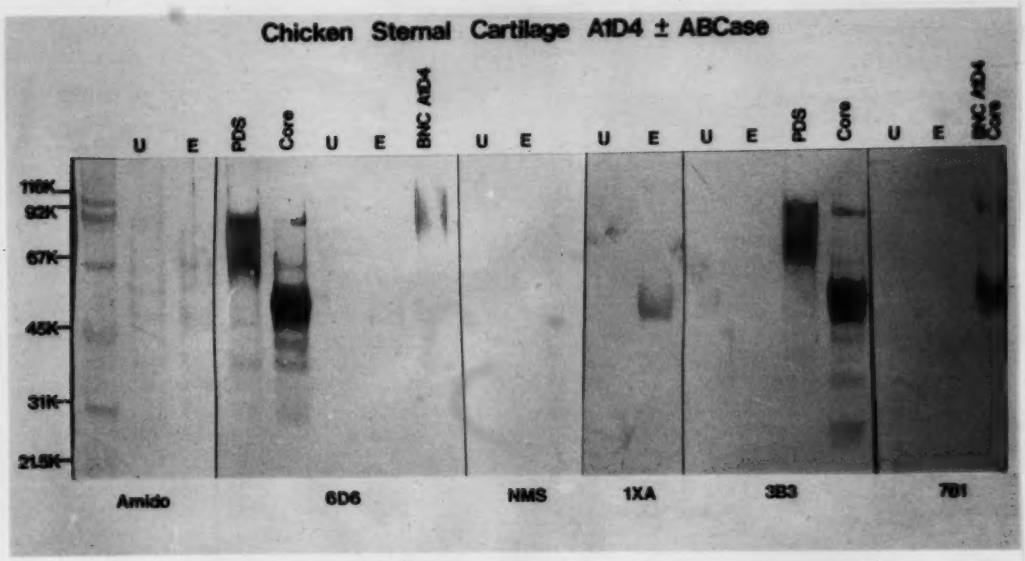

Plate 23.

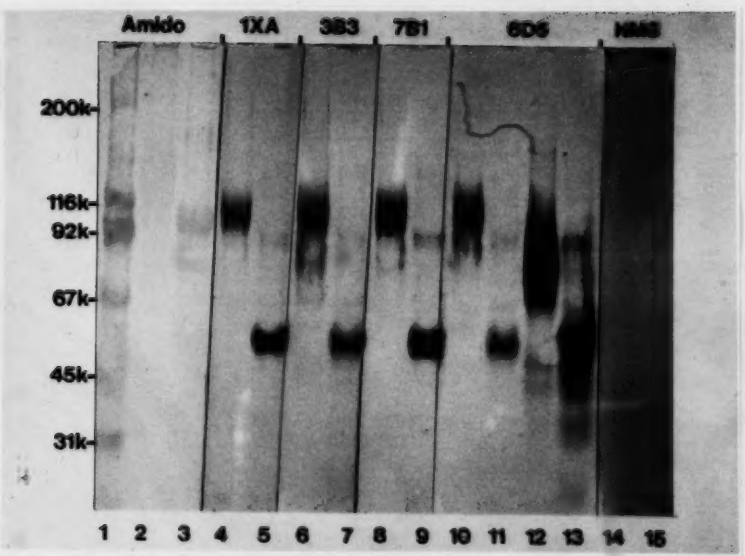

Plate 24.

Plate 25. Immunoblot of bovine dental pulp PG1. The sample was added to 0.1M sodium acetate, 0.1M Tris HCl, pH 7.4 buffer and dissolved by boiling for 15 min. Chondroitinase ABC (0.1 unit/mg sample) was added to one half of the sample and both portions were subsequently incubated for 55 min at 37°C. Both portions were diluted with appropriate volumes of SDS-PAGE sample buffer and boiled for 5 min. 15 μg of undigested PG1 was applied to lanes 2, 4, 6, 8, 10 and 14, while 15 μg of the enzyme treated PG1 were applied to lanes 3, 5, 7, 9, 11 and 15. Bovine skin PDS (300 ng protein) and its (ABC)core preparation (100 ng protein) were applied to lanes 12 and 13, respectively. The samples were transferred from a 5% gel and stained with Amido black or immunochemically as indicated

Plate 26. Immunoblot of fractions from peaks 1, 2 and 3 of PDS (ABC)core preparation chromatographed on Sephacryl S-300. 2 μl aliquots of fractions 34 (peak 1), 41 (peak 2) and 48 (peak 3) were diluted with 30 μl SDS-PAGE sample buffer lacking 2-mercaptoethanol, boiled for 5 min and applied to lanes 2-4, respectively. The same sample volumes diluted in sample buffer containing 10% 2-mercaptoethanol were boiled and applied to lanes 5-7, respectively. 40 ng (protein) of bovine skin PDS and its (ABC)core preparation were applied to lanes 8 and 9, respectively. Samples were transferred to nitrocellulose from a 5% gel and stained with Amido black or immunochemically with 6D6 ascites fluid (1:1000) as indicated.

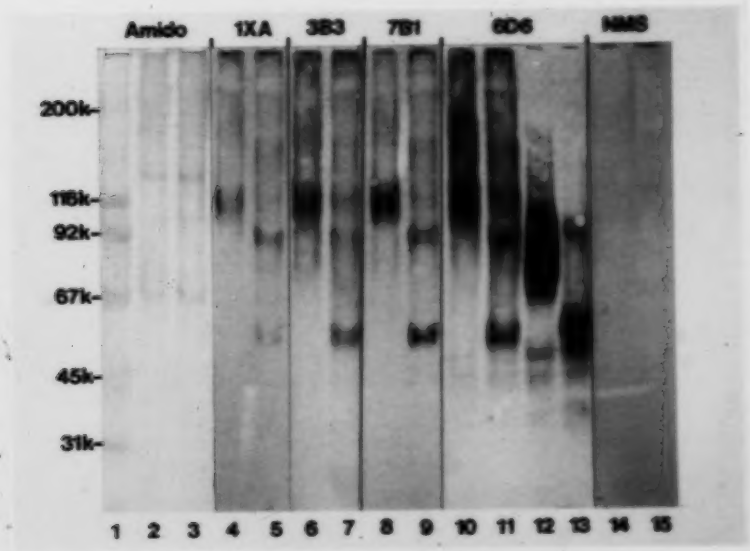

Plate 25.

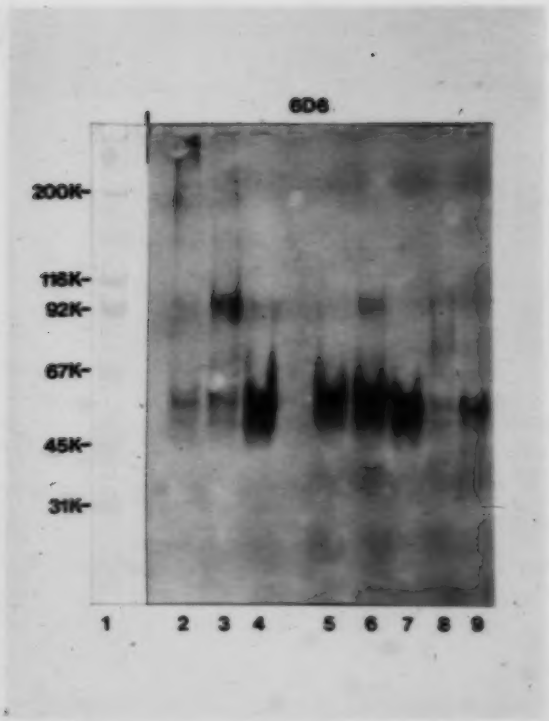

Plate 26.

Plate 27. Immunoblot of bovine skin PDS and its (ABC)core preparation. Chondroitinase ABC (0.1 unit/mg sample) was added to one half of a PDS sample, previously dissolved in a 0.1M sodium acetate, 0.1M Tris HCl, pH 7.4 buffer, and both portions of the sample were incubated for 50 min at 37°C. The undigested sample was applied to lanes 2 and 4 (2 μg), 6 and 8 (100 ng) and 10 and 12 (400 ng). The enzyme treated sample was applied to lanes 3 and 5 (2 μg), 7 and 9 (100 ng) and 11 and 13 (400 ng). Samples applied to lanes 2 & 3, 6 & 7 and 10 & 11 were diluted in SDS-PAGE sample buffer containing 10% 2-mercaptoethanol and boiled for 4 min. Samples in lanes 4 & 5, 8 & 9 and 12 & 13 were diluted in SDS-PAGE sample buffer containing 8M urea, 10% SDS and 10% 2-mercaptoethanol and boiled for 4 min. (The final concentrations of urea and SDS in lanes 4 & 5 were 4M and 5%, respectively; in lanes 8 & 9, 7.8M and 9.6%, respectively; and in lanes 12 & 13, 5M and 6%, respectively). Samples were transferred from a 7% gel and stained with Amido black or immunochemically with 6D6 ascites fluid as indicated.

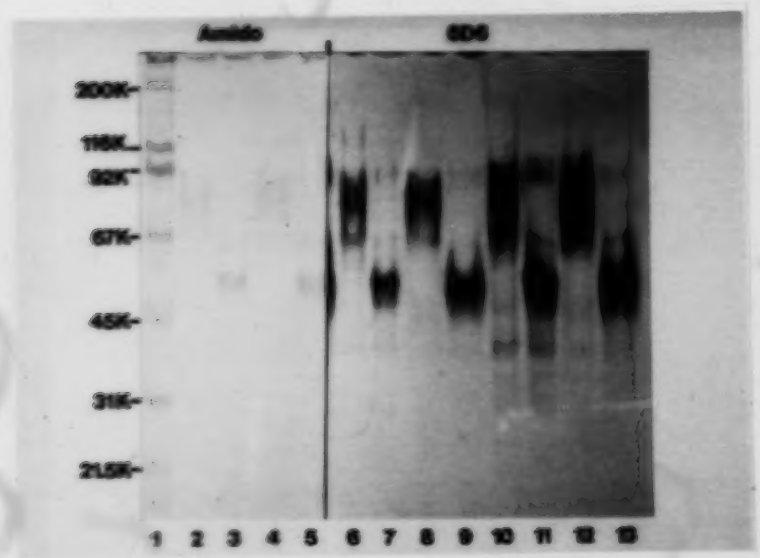

Plate 27.

Plate 28. Immunohistochemical localization of the protein core of proteodermatan sulfate in adult bovine skin. (a) Formalin-fixed 5 μm section stained for peroxidase after an overnight incubation at 6°C with 6D6 supernatant fluid (diluted 1:10), linking antibody (45 min) and mouse peroxidase anti-peroxidase (30 min). (b) Higher magnification of subepidermal zone of same section. (c) Higher magnification of connective tissue surrounding a hair follicle and blood vessels of same section. (d) Serial section to (a) using normal mouse serum (diluted 1:100). The horizontal bar in figures (a,c and d) = 100μm; in figure (b) the bar = 10μm.

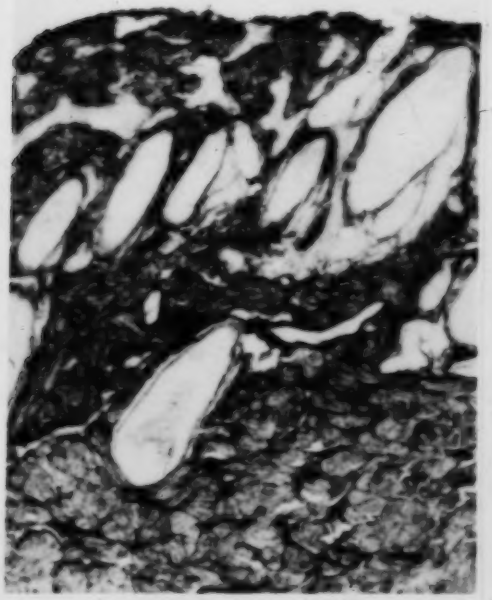

Plate 28a.

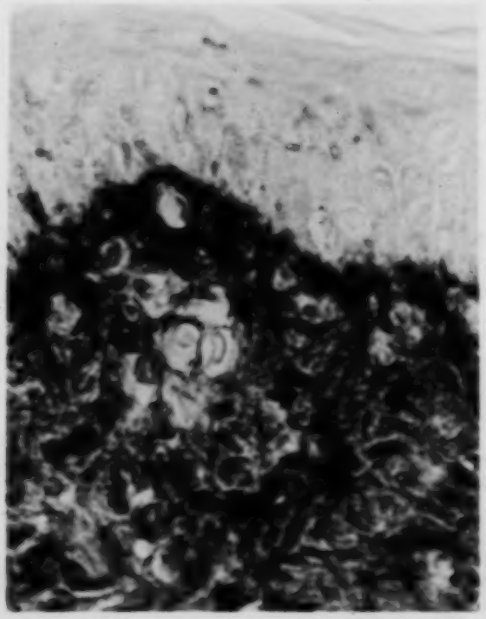

Plate 28b.

Plate 28c.

Plate 28d.

Plate 29. Immunohistochemical localization of the protein core of proteodermatan sulfate in fetal calf skin. (a) Formalin-fixed section of fetal calf skin estimated to be 130 days of age, stained for peroxidase after overnight incubation with a 1:10 dilution of 6D6 supernatant fluid. (b) Section of the same tissue in which the enzyme reaction was allowed to proceed for an extended period of time. (c) Fetal calf skin control using normal mouse serum diluted 1:100. Horizontal bar = 100 μm.

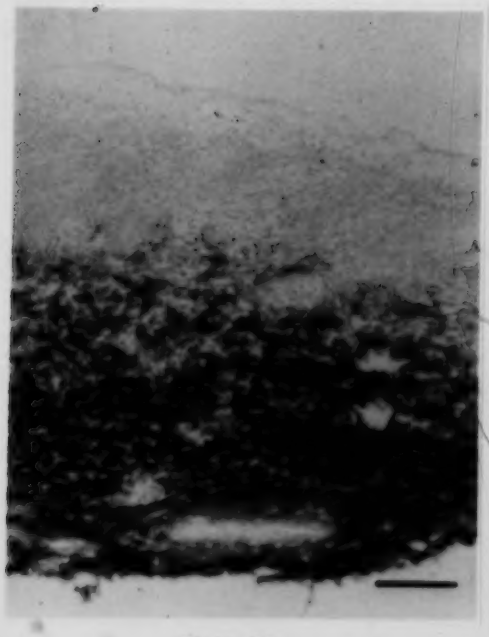

Plate 29a.

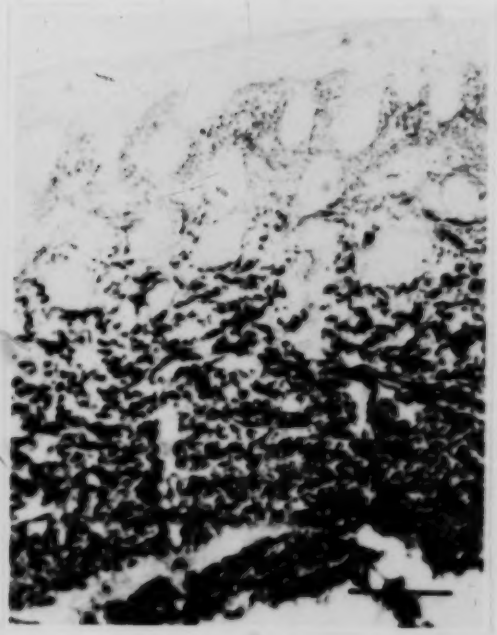

Plate 29b.

Plate 29c.

Plate 30. Immunohistochemical localization of the protein core of proteodermatan sulfate in fetal calf skin of different stages of development. Formalin-fixed 5 μm sections of skin from 4 different animals were incubated overnight at 6°C with 6D6 supernatant fluid (diluted 1:10) and stained for peroxidase using the peroxidase anti-peroxidase method. Sections a-d demonstrate progressive development of hair follicles. Horizontal bar = 100 μm.

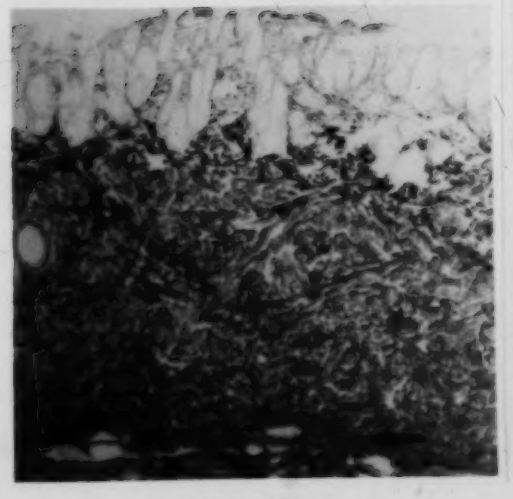

Plate 30a.

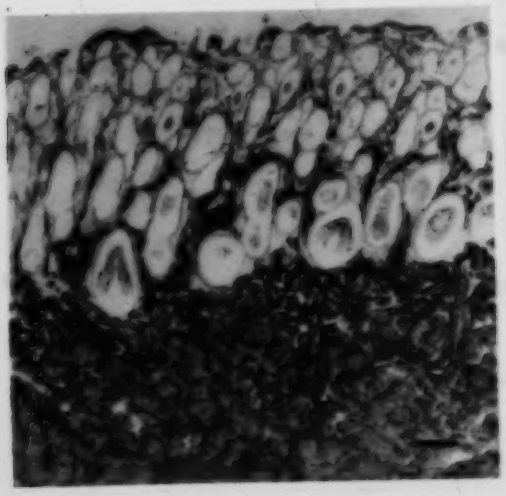

Plate 30b.

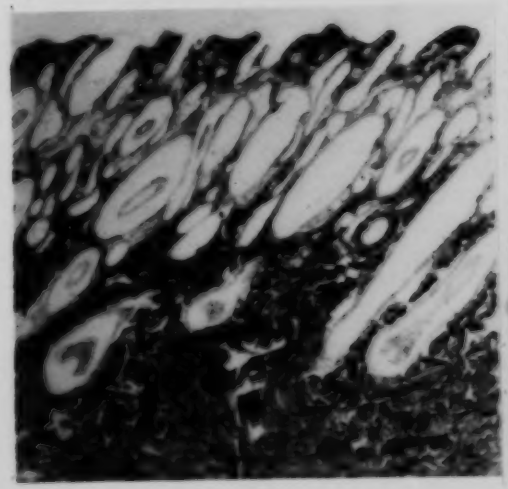

Plate 30c.

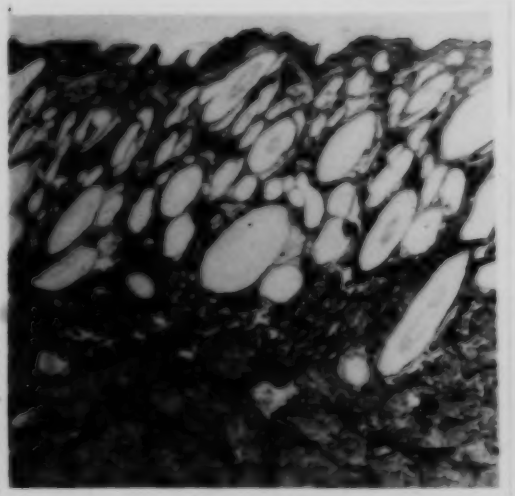

Plate 30d.

Plate 31. Localization of the protein core of proteodermatan sulfate using the peroxidase anti-peroxidase method in a formalin-fixed section of adult human skin that had been incubated with 6D6 supernatant fluid (diluted 1:10) overnight at 6°C and stained for peroxidase. Horizontal bar = 10 μm.

Plate 32. Formalin-fixed 5 μm section of bovine gingiva similarly stained for peroxidase after incubation with 3B3 supernatant fluid diluted 1:2. Horizontal bar = 100 μm.

Plate 33. (a) Frozen, unfixed section of human gingiva incubated overnight with 1XA supernatant fluid (diluted 1:2) and stained for peroxidase. (b) Section of the same tissue incubated with with 7B1 supernatant fluid. Horizontal bar = 100 μm.

Plate 31.

Plate 32.

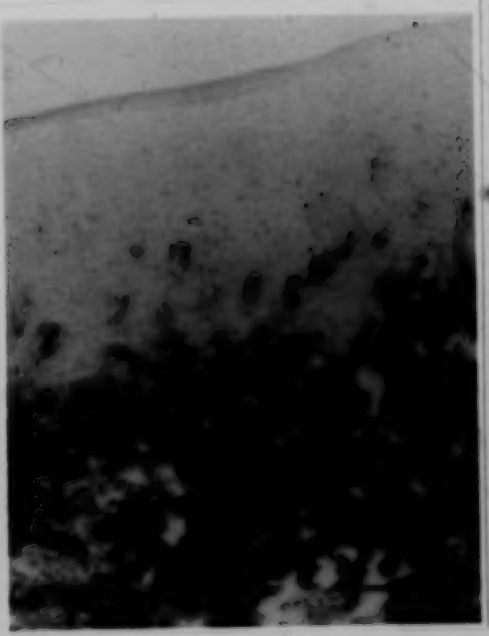

Plate 33a.

Plate 33b.

Plate 34. Immunohistochemical localization of the protein core of proteodermatan sulfate in a formalin fixed section of adult bovine tail tendon that had been incubated overnight with 7B1 supernatant fluid (diluted 1:2) and stained for peroxidase.

Plate 35. Formalin fixed section of bovine dental pulp from an unerupted incisor stained for peroxidase after incubation with a 1:10 dilution of 6D6 supernatant fluid.

Plate 36. (a) Fixed section of adult bovine nasal cartilage and underlying nasal tissue that had been incubated with 3B3 supernatant fluid (diluted 1:2) and subsequently stained for peroxidase. (b) Same tissue stained with Alcian blue to highlight the location of the hyaline cartilage matrix.

Horizontal bar = 100 μm

Plate 34.

Plate 35.

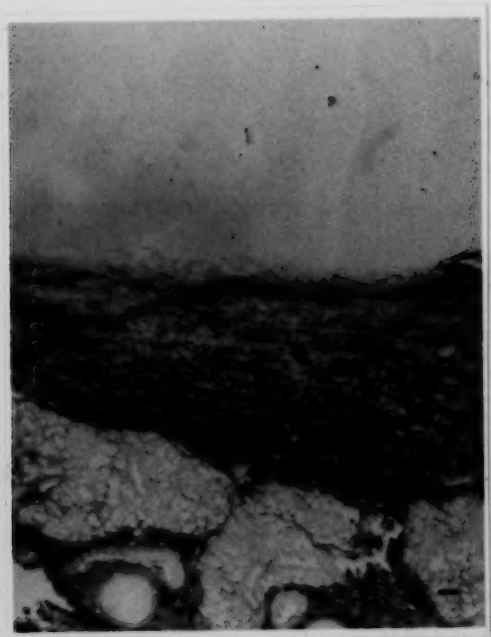

Plate 36a.

Plate 36b.

Plate 37. Immunohistochemical localization of the protein core of proteodermatan sulfate on the surface of adult bovine tail tendon collagen fibrils. Tendon pieces were incubated overnight with MAb supernatant fluid and, once stained for peroxidase using the peroxidase anti-peroxidase method in the usual manner, were embedded in Spurr's resin. Thin sections were examined using a Philips 410 transmission electron microscope. (a-c) Sections (incubated overnight with 6D6 supernatant fluid) that show the arrangement of the (osmicated) oxidized diaminobenzidine precipitate particles on fibrils sectioned longitudinally. (d) Tendon collagen fibrils (that had been incubated with 7B1 supernatant fluid) sectioned more obliquely to demonstrate that the immunochemical reaction products did not penetrate the spaces between closely packed fibrils. Horizontal bar = 0.1μm.

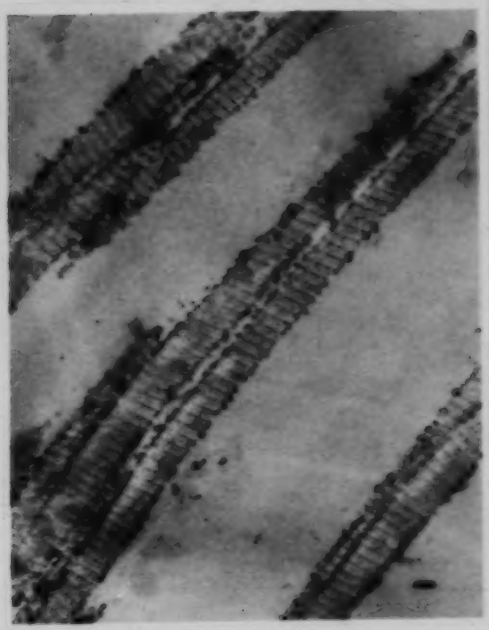

Plate 37a.

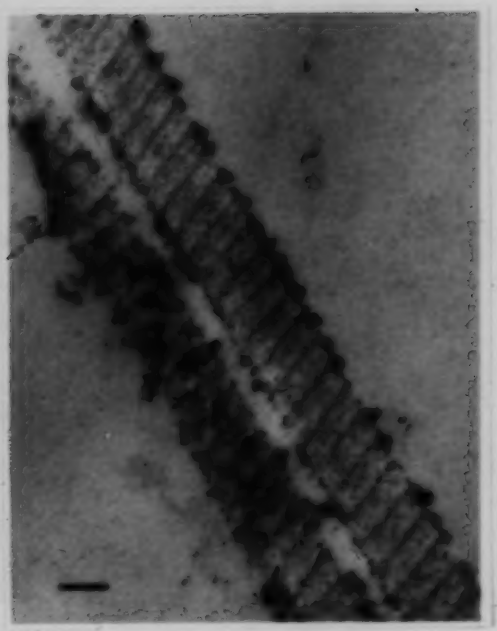

Plate 37b.

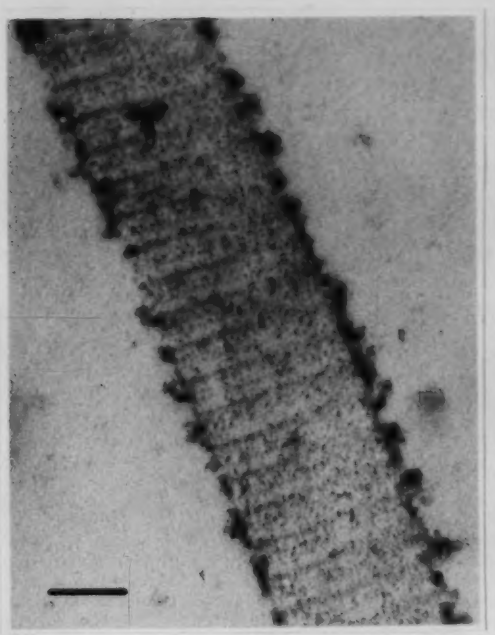

Plate 37c.

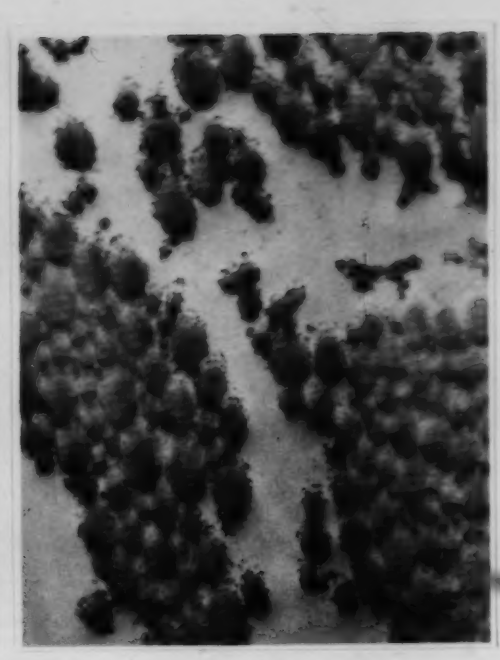

Plate 37d.

Plate 38. Immunohistochemical controls for the ultrastructural localization of the protein core of proteodermatan sulfate in bovine tail tendon. Thin sections of bovine tail tendon were incubated with: (a) 1:100 dilution of normal mouse serum in 30% normal goat serum in TBS; (b) 30% normal goat serum in TBS and (c) 6D6 supernatant fluid that had been preadsorbed with bovine skin PDS. Horizontal bar = 0.1 μm.

Plate 38a.

Plate 38b.

Plate 38c.

Plate 39. Immunoelectron microscopical localization of the protein core of proteodermatan sulfate on the surface of mature bovine tail tendon collagen fibrils using ferritin-conjugated secondary antibodies. (a-b) Thin sections of tendon that had been incubated with a 1:10 dilution of 6D6 supernatant fluid overnight at 6°C. (c) Normal mouse serum (diluted 1:500) control. x 133,000.

Plate 39a.

Plate 39b.

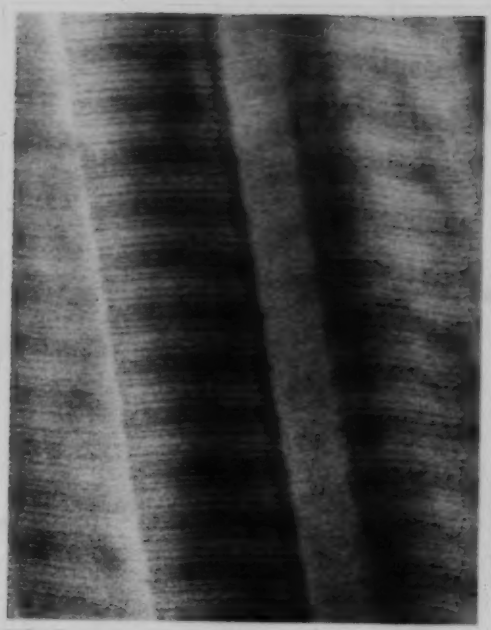

Plate 39c.

Plate 40. Ultrastructural localization of the protein core of proteodermatan sulfate on the surface of bovine tail tendon collagen fibrils using the immunoferritin method in conjunction with 7B1 supernatant fluid (diluted 1:2). x 133,000.

Plate 40a.

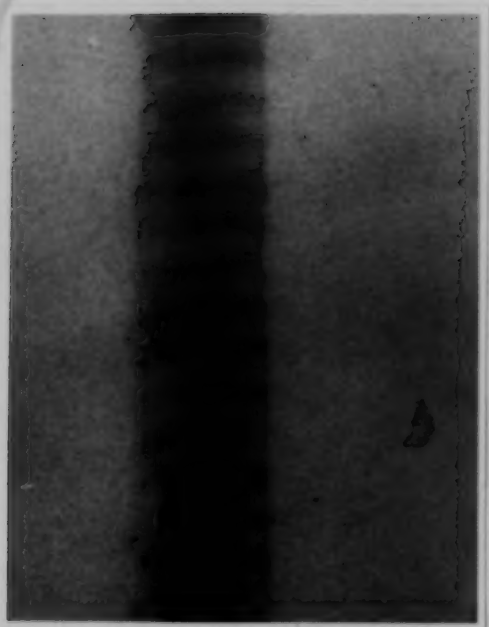

Plate 40b.

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